ZACHARY T. KELLEHER. The cloning of the Fanconi's anemia gene. (Under the direction of Dr. Jean-Michel Vos).

The aim of this project was to pinpoint the location and ultimately the gene code of one of at least four possible genes of Fanconi's anemia, a human "DNA repair" disorder. In the first part of the study, microcell-mediated chromosome transfer, an established procedure, was used to transfer a normal tagged human chromosome #20 (the reputed location of the FA-A gene, carried in an A9 mouse cell line) into FA-group A lymphoblasts and fibroblasts at which point they would be tested to see if the uptake of this chromosome corrects two prominent manifestations of the FA defect, namely its inherent chromosomal instability and its hypersensitivity to DNA cross-linking agents. Success in this venture was elusive.

In the second part of the study, the first steps of a proven method of gene cloning was tried involving first, the transfection of a cDNA library, carried in an Epstein-Barr virus (EBV) vector into an FA-group B cell line, and then pulling out the FA-B gene from these cells by treating them with DNA cross-linking agents and selecting for ones which have lost this hypersensitivity. Three different methods of transfection, electroporation, lipofection and endocytosis-fection, were tested to discover which was the most efficient at transferring plasmid DNA into the FA group B cells. This group, which is one of the most sensitive of the four known FA cell lines, was transfected, in transient assays, most efficiently via endocytosis-fection. However, the attempt to select for long term maintenance of plasmid DNA was unsuccessful for FA-B cells using any of the transfection methods. However, successful maintenance of the cDNA-containing plasmid by FA-A lymphoblasts was obtained. Modifications are being made to circumvent the problem with the former cell line while work continues to build on the success with the latter cell line.

ACKNOWLEDGMENTS

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CHAPTER I: INTRODUCTION

The integrity of the genetic material of all living cells is constantly being challenged by "outside" environmental agents such as heat, radiation or mutagenic compounds, by DNA modifiers generated by cellular metabolism and by the inherent chemical instability of DNA. Alterations in the genetic material could, if left uncorrected, be deleterious to the cell by affecting the replication process, in the case of cross-links between DNA strands, or by altering important genes, by either the insertion, deletion or substitution of base pairs within the genome. Fortunately, cells have developed elaborate and efficient methods of repairing altered or damaged DNA to combat the daily onslaught of harmful agents.

Out of the many repair systems, the most classic is "excision repair" which involves a number of factors acting cooperatively to restore altered DNA to its undamaged form. These factors must perform a number of functions including recognizing the damaged DNA, binding to the lesion, "nicking" or cutting the DNA backbone around the lesion, excising or removing the nucleotides between the nicks, replacing the nucleotides, and finally ligating the newly synthesized DNA to the old (figure 1). At the present time this system is best characterized in procaryotes, especially in the UvrABC repair in the bacteria Eschericia coli, where many of the DNA repair genes have been identified and cloned *(38)*.

While excision repair can be used to repair damage to one strand of DNA, more complicated mechanisms, such as bypass repair and recombination, must be used to repair damage involving both strands. For this type of damage, the DNA lesions are bypassed during replication and then, via recombination with the homologous strand of DNA (which acts as the new template), restored to its original form. Though the basic mechanisms of excision repair, such as the recognition and replacement of the damage, as well as the resynthesis of new DNA, are involved in interstrand cross-link repair in eucaryotes, the exact mechanisms of repair as well as the many components involved in it remains poorly characterized*(57)*. The most progress in characterizing the mechanisms and enzymes

FIGURE 1: DNA EXCISION REPAIR IN PROCARYOTES

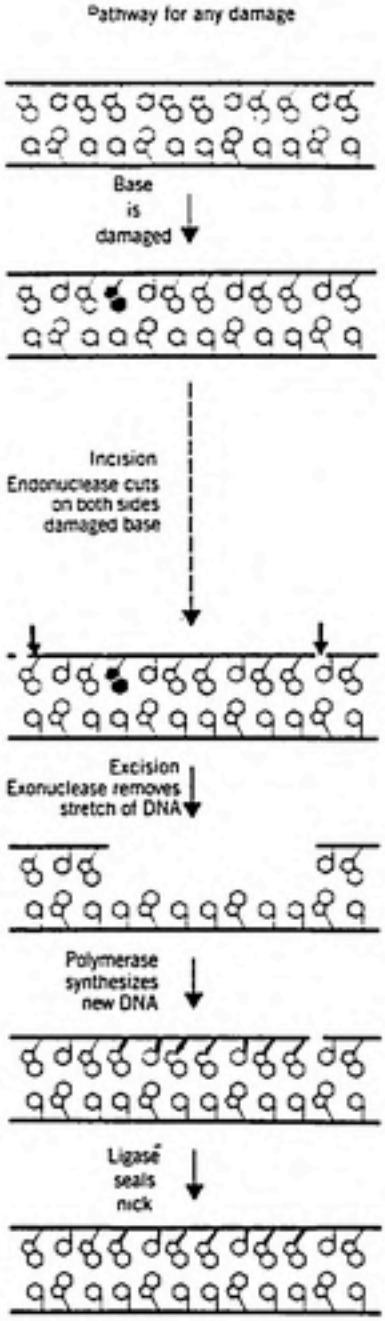


Figure 19.8
Excision-repair removes and replaces a stretch of DNA that includes the damaged base(s).

Right. The general pathway for repair in *E. coli* involves cutting on both sides of the damaged base, followed by excision.

(Reprinted from Genes IV, p. 379, Lewin, B., Oxford, England, (1990)

involved in repair in eucaryotes have come in the study of yeast where many radiation inducible genes (RAD) have been found *(55)*.

Further study on a cellular process as crucial and yet as poorly understood as DNA repair is imperative for a number of reasons, many of which are elucidated by Vos *(57)*. With the increase in potential DNA damaging agents being released into the environment each year it is especially important to know how these agents are affecting living organisms on a cellular level and how these effects are handled by the cells of the organism. This knowledge could potentially affect treatment of those exposed to these harmful agents as well as on the manner in which these agents are released into the environment. It can also help to delineate the role of repair in the complex and pathogenic multicellular processes such as cancer and aging.

Fanconi's anemia (FA) is a recessive, inherited genetic disease which has been characterized as a "repair disorder" along with other defects such as Xeroderma Pigmentosum (XP), Ataxia telangiectasia, (AT), Bloom's syndrome (BS), and Cockayne's syndrome (CS). The fact that it is inherited makes it a stable system in which to study DNA repair. The exact mechanisms of the FA defect is unknown, though cells from FA patients apparently are deficient in their ability to repair cross-links of their DNA and are genetically unstable. The defect is thought to be the result of an altered endonuclease responsible for the initial incision step of repair. However, four different types of FA have been found each of which could involve a separate factor.

CHAPTER II: LITERATURE REVIEW

Fanconi's Anemia.

What is Fanconi's Anemia?

Fanconi's anemia (FA) is a rare autosomal recessive disorder which is characterized by some or many of the following: a predisposition to acute myelegenous leukemia and other malignancies, bone marrow failure, congenital abnormalities which include skeletal deformities such as absence of thumb and radii, intrauterine growth retardation, short stature, hyper pigmentation and pancytopenia *(18, 24, 49)*.

Cells from persons with FA are characterized by a higher than normal rate of spontaneous and induced level of chromosomal breaks, a hypersensitivity to DNA cross-linking agents, an increased sensitivity to oxygen and a perturbed cell cycle *(3, 27, 29, 46)*. The manifestations of any of these defects varies widely between patients both clinically, with differences in the incidence, type, onset and severity of the malformations, and biochemically, with wide variations in sensitivities to DNA damaging agents and level of chromosomal aberrations. There has of yet been no correlation found between the severity of the clinical symptoms and cellular deficiencies *(4)*.

This extreme heterogeneity of FA symptoms is a factor which must be considered whenever analyzing data of this disorder. It also may indicate that the FA defect is a complicated one, involving more that just DNA repair. At present it is believed that there are at least four different genes or gene parts which are responsible for the FA defect. Through complementation studies, four different complementation groups of FA cells have been established, labeled, respectively, A-D *(15, 52)*. (Complementation groups are determined by fusing cells from different FA patients via somatic cell fusion and checking the cross-linking sensitivity of the subsequent products. If there were only one gene responsible for the FA defect then the hypersensitivity of the fused cell line would be expected, for the most part, to be similar to that of the original cells lines

because each line would contribute two defective FA genes. However, when cells from certain FA patients are fused, the subsequent sensitivity of the resulting cell line is similar to that of normal cells, indicating that a correction of the defect has taken place. This could be explained if there were more than one defective gene or gene part involved. The defective gene of one cell line is masked by a normal copy of that same gene or gene part contributed by the second cell line and vise versa. Cells in this latter category are said to be in different complementation groups since they complement each other and apparently contain different mutations. Cells belonging to the former category are said to be in the same complementation group since they cannot complement each other and therefore share the same defect.

What are the primary characteristics of FA cells?

Though there is wide variation in the phenotype between FA cells there are certain characteristics that are consistently found among them. In trying to determine how best the gene might be cloned, the most salient features of the defect have been examined. These characteristics can ultimately be exploited to uncover the nature of the FA impairment and the mutated genetic code which underlies it.

There are five primary manifestation of the FA defect on the cellular level. The first and one of the most obvious is the increased rate of spontaneous chromosomal aberrations (CA). These were first characterized by Schroeder in 1967 and have since been described by numerous other researchers *(8, 15, 43, 56)*. The types of aberrations seen most often are chromatid gaps and breaks though the level of sister chromatid exchange (SCE) has been shown normal in FA cells *(35, 36)*.

The extent of spontaneous CAs varies between the cells of different patients as well as in cells from the same patient over time *(8, 49)*. Additionally, the CA rate varies among cells in the different complementation groups with FA-B cells demonstrating a higher rate of spontaneous breakage than either FA-A or FA-D cells. FA-C cells, one of the newest FA cells lines has yet to be characterized for spontaneous or induced chromosomal breakage *(13, 15, 16)*.

The second is that FA cells have an increased rate of induced chromosomal aberrations (over that of the spontaneous level) whenever

exposed to DNA cross-linking agents. This is in contrast to normal cells which show no such characteristic increase when treated with similar doses. This induced rate of chromosome breakage in FA cells is one of the most consistent features of the disease and indeed, it is used as the pre-eminent characteristic in the diagnosis of the disorder *(4, 8, 15, 16, 43, 56)*.

The third most salient feature of the FA defect is the sensitivity of FA cells to DNA cross-linking agents. Cross-linking agents are compounds which can intercalate into DNA, covalently linking either two adjacent base pairs (intra-strand cross-links) or two base pairs on opposite strands (inter-strand cross-links). In the process of characterizing the FA defect, cells from FA patients have been treated with a number of different DNA-damaging chemicals such as mitomycin C (MMC), diepoxybutane (DEB), psoralen and UVA light (PUVA), cis-diamine-dichloro-platinum II (cis-Pt (II)), other mono- and polyfunctional alkylating agents, nitrogen mustard, and ionizing radiation *(3, 7, 16, 27, 43, 46)*. The majority of these studies have found that FA cells have a deficiency in the repairing the damage caused by those compounds which form DNA cross-links, though there are studies which dispute this *(19, 32, 42)*. These latter studies might be explained by the extreme heterogeneity that exists between the various FA complementation groups, which were not characterized in the reports sited. Indeed, the FA-D group, represented by the cell line HSC 62 and most likely FA-145, has been shown to be only slightly more sensitive to cross-linking agents than are normal cells both in the repair of these cross-links *(40)* and survival after DNA cross-linking treatment *(16, 27, 41, 60)*.

Additionally, different cross-linking agents work by different mechanisms and may require different repair pathways. Thus, the extent of cross-linking sensitivity may vary depending on the agent used. It has been observed that FA cells are more sensitive to PUVA than to MMC by an order of magnitude based on the EC10, the effective concentration of cross-linking agent which inhibits growth to 10% of normal, when comparing the difference in sensitivities between normal and FA cells. FA cells treated with MMC are 1-11 times more sensitive than normal cells while with PUVA the difference is 15-110 times (personal data). It is known that PUVA interacts primarily with pyrymidines whereas MMC (and nitrogen mustard) form cross-links between purines, specifically on the O_6 of guanine *(41)*. At present it is generally accepted that while FA

cells do have the ability to repair DNA cross-links, they do so at a lower efficiency than normal cells *(27, 40, 41)*. This defect in cross-link repair along with the genetic instability are the strongest evidence that FA as a DNA repair disorder.

The fourth defect manifest in FA cells is their sensitivity to oxygen. Joenje and colleagues have shown conclusively that the level of chromosomal aberrations in FA cells is dependent on the level of oxygen in the ambient environment. When FA cells were exposed to reduced levels of oxygen (0.0-5.0%) a decrease in the level of breaks was observed. Conversely, when these cells were placed in an environment with an increased level of oxygen compared to normal levels, an increase of chromosomal breaks was observed *(29, 30)*. This has lead some to propose that the primary defect in the FA disorder is not, as generally believed, a defect in the DNA repair system but rather a deficiency in the cells ability to detoxify reactive oxygen species *(29, 30, 31)*.

Finally, FA cells have disturbed cell cycle manifest in a prolonged S and G2 phase. Weksberg et al. (1979) have found that the increased doubling time in FA cells is due to an increased generation time as well as a higher rate of cell death. Dutrillaux (1982) found that it took FA cells on average 32-40 hours to pass through one full cycle, while the time for normal cells was 28 hours *(17, 47, 60)*.

What is the nature of the FA defect?

Aside from the clinical aspects of the disease, the primary focus, biochemically, on the FA disorder has been on the defect in repair, though, as mentioned, this is not the only defect in this syndrome. While it has been conclusively demonstrated that FA cells are slower in their repair of interstrand cross-links and have a higher rate of chromosomal breakage compared to normal cells, the exact mechanism of this process has yet to be fully elucidated though a few theories have been proposed which find experimental support. There has been growing evidence that the defect in repair in FA cells involves the first incision step of the cross-link repair process, more specifically, that there is an alteration in one of the endonucleases which perform the initial incision step, retarding its ability to do its job *(21, 22, 25, 34, 41, 44)*. Sakaguchi et al. (1992), demonstrated that a Chinese hamster mutant (mus308), which is analogous

to FA cells in its sensitivity to cross-linking agents and its high level of spontaneous chromosomal aberrations, has a mutation which results in an defective endonuclease. More specifically, Lambert et al. (1992) have isolated modified endonucleases from both FA-A and FA-D cells (the latter which they refer to as FA-B). The defective endonuclease in FA-A cells recognizes psoralen intercalation's and interstrand cross-links and takes part in the initial incision step of the repair process. In their studies, it exhibited 25% the activity of the normal endonuclease. The altered nuclease in the FA-D cells recognizes psoralen monoadducts and has approximately 50% of the activity of the normal endonuclease. These findings were later strengthened when the defects in each of these FA groups was corrected with the insertion of the respective normal endonuclease *(34, 44)*. These finding confirm earlier studies which found FA-A cells to be defective in the first incision step of the cross-link repair process and that FA-D cells have a defect in the repair of monoadducts formed by psoralen and UVA light *(6, 41, respectively)*.

Though an altered endonuclease may be responsible for the diminished repair capacity in FA cells, it does not necessarily follow that the mutated FA gene(s) codes for such an enzyme. Such an claim would fail to account for the observed oxygen sensitivity and cell cycle disturbance of FA cells. At present, no explanation has been proffered nor empirical evidence found which might adequately account for the all of the known FA defects. This may indicate that the mutated gene responsible for the FA defect codes for a multipurpose enzyme and not just an enzyme involved in DNA repair.

CLONING THE FA GENE

Reasons for cloning the FA gene.

Their are two basic routes that can be used to uncover the FA defect, namely the biochemical approach, which focuses on the actual mechanisms of a system and the components involved therein, as was used by Lambert et al, (1992) and Sagaguchi et al. (1992) who examined the modified endonucleases in FA or "FA-like" cells, or the genetic approach, which tries to uncover the code of the mutated gene responsible for the disorder, like Strathdee et al. (1992) who have successfully cloned the FA-group C gene. This project follows the latter of these routes *(34, 44, 53)*.

The genetic approach has the advantage of ultimately discovering the code of the mutated gene which may lead to the discovery of the biochemical mechanisms of the gene product, though finding the gene doesn't guarantee discovering the defective protein. However, if the gene is cloned, its homology to any other known gene can be checked. If it shares homology with other characterized genes, the information on these genes might help to further uncover the nature of the FA defect and what role repair plays in it. Or, as in the case of the FA-C gene, it could turn out to be novel or uncharacterized, in which case the discovery of the protein and mechanism of action may be even more valuable.

A second advantage to discovering the code of the FA gene is for its potential use in gene therapy. If a normal copy of the FA gene could be delivered to certain target cells in the body of a FA patient, as in systems which are now being developed in cystic fibrosis and other diseases, this would be an enormous benefit to those people with the disease who now can rely solely on bone marrow transplants for the treatment of the disease. There are indeed systems like this being developed at the present time*(54)*.

A third advantage of using the genetic approach is that once the gene is found, it can be used, in the case of the FA gene, for more sensitive diagnosis of the disease. Though the present assays, based on sensitivities of the cells of FA patients to DEB, are adequate in diagnosing FA, more precise determinations could be made if the gene was known.

There are two parts to this project; The goal of the first part is to confirm the assignment of the FA-A gene to normal human chromosome (HC) #20 by means of microcell mediated chromosome transfer (MMCT). The successful completion of this goal would eliminate a large portion of the human genome from consideration as a site for this gene, which stand as yet, uncloned. The goal in the second part of the project is the actual cloning of the FA-B gene using a new system used to clone the FA-C gene. The successful completion of this project would unlock the code of a second FA gene and shed additional light on the FA disorder.

Project I: Confirming the putative location of the FA-A gene.

Justification

In looking for effective methods in uncovering the location of the FA gene, one strategy is to examine the paths taken by those exploring other "DNA repair disorders" such as Xeroderma Pigmentosum (XP), Ataxia telangiectasia (AT), Bloom's Syndrome (BS), or Cockayne's Syndrome (CS), some of which are farther along in the cloning of the respective genes *(1)*.

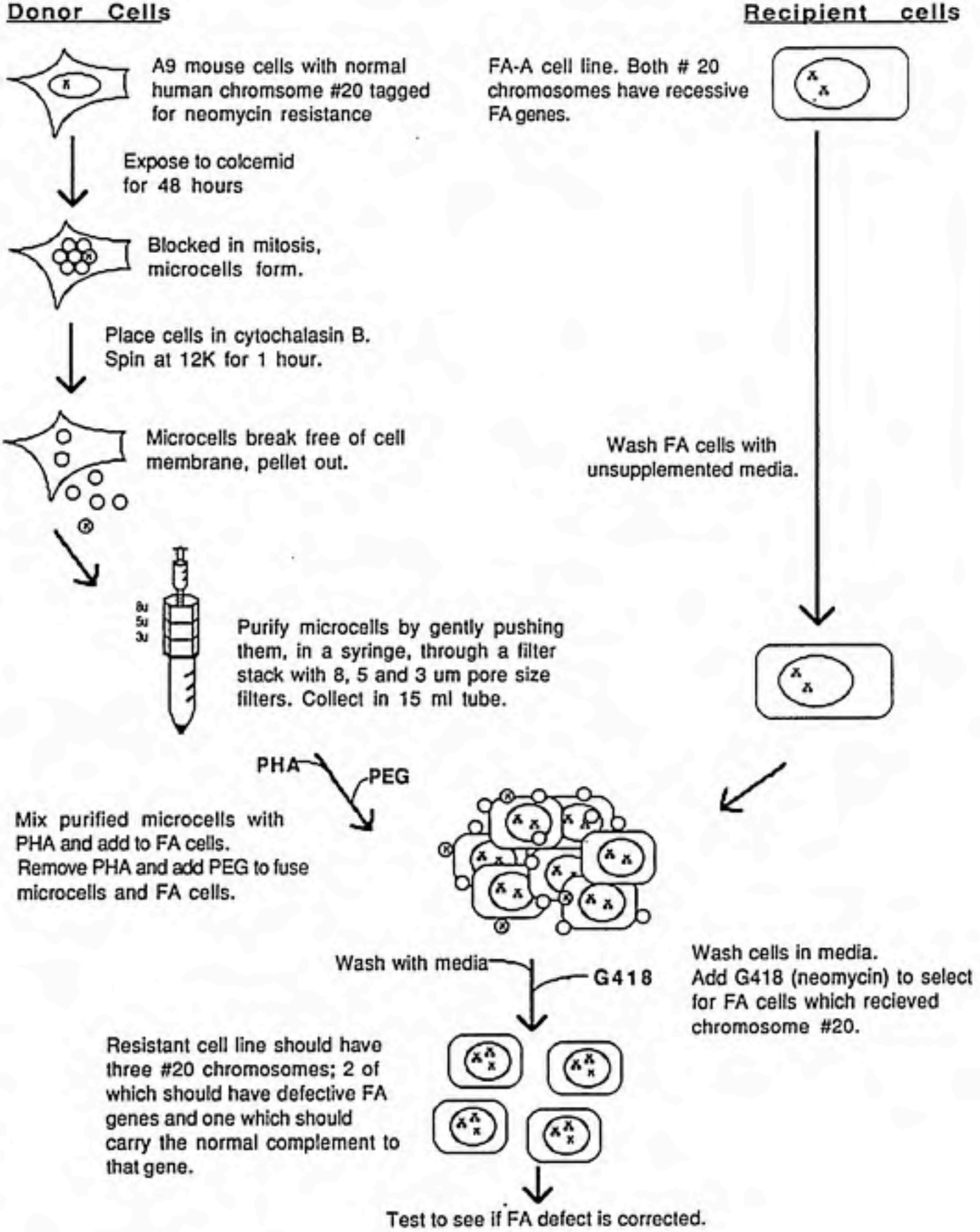
There are three key studies involving other "DNA repair" disorders which provided the basis for this project. In the attempt to find the chromosomal location of an AT gene, Gatti et al. (1988) performed a genetic linkage analysis in 31 families with AT group D (AT-D) affected individuals. From this study they estimated the AT-D gene to be on chromosome 11 q22-23 *(23)*. Following this study, Komatsu (1990) inserted a normal human chromosome 11 (HC 11) into AT-D cells via MMCT and achieved correction of the defect, thus confirming the previous study *(33)*. In earlier studies, the MMCT procedure was also used to correct the XP-A defect by insertion of a normal, however, transposed human chromosome *(50)*.

As with the Gatti study, Mann et al., (1991) performed a genetic linkage study on 34 families which had members affected with FA and estimated that the FA-A gene is located on HC 20q *(39)*. The next step in uncovering the chromosomal location of the FA-A gene, following the path taken with the AT-D research, was to insert HC 20 into FA-A cells to determine if correction takes place.

Approach

To test the hypothesis that the FA-A gene resides on HC20, MMCT was used to transfer HC20 into FA-A cells. MMCT is an established method of chromosome transfer and is used often to transfer human chromosomes into rodent cell backgrounds or visa versa for the purpose of gene complementation and for chromosome mapping *(20, 45, 48, 59)*. In the MMCT procedure, microcells, or enucleated sacs containing various numbers of chromosomes, are formed by the addition of a mitotic blocking

FIGURE 2: MICROCELL-MEDIATED CHROMOSOME TRANSFER.



agent, such as colcemid, to the cells for an extended period of time (24-72 hours, see figure 2). These micronuclei are then collected by first placing the cells in a solution of cytochalasin B, an agent which destabalizes the microtubules, causing a loosening of the cell membranes, then spinning them at a high velocity whereby the microcells escape the confines of the cell membrane and pellet out.

The microcells are purified via filtration then transfected into a given cell line by first treating them along with the recipient cells with phytohemoglutinin (PHA) which helps the microcells to attach to the recipient cells, and then with polyethylene glycol (PEG) which weakens the cellular membrane and allows the microcells and their contents to be taken up by the cells. There is usually a selectable marker, such as a hygromycin or neomycin resistance gene, on the inserted human chromosome. This allows for the selection of cells which take-up this chromosome. In this experiment, a mouse cell line containing a single human chromosome (#20) tagged for neomycin resistance, was used as the donor cell line to be fused to one of four different cell lines characterized as belonging to FA group A.

Once this was successfully completed, the next step would be to test the cells for correction of the FA defect by observing the spontaneous and induced levels of chromosomal breakage as well as the cross-link sensitivity of the new cell line. Correction of both of these parameters would be a positive indication that the FA-A gene was on HC 20. Correction of only one of these parameters, a partial complementation, would indicate, among other things, that part of the FA gene was missing from the inserted chromosome or that there was an overexpression of a protein which compensates for the one defective in the FA system. A failure to achieve correction in either of these two parameters might indicate that the FA-A gene is located on another chromosome though this result might also be observed if the gene was masked or altered or had become disassociated from the marker.

Project Two: The Cloning of the FA-B Gene

The scope of the second project was wider and more involved than that of the first. In this project the objective was to recover and characterize an FA gene, rather than the merely determine the its chromosomal location.

Justification:

Over the past decade there have been numerous attempts to clone the FA gene(s) and many methods using a variety of DNA types have been used, ranging from transfecting FA cells with wild type genomic DNA *(10)*, injection of HeLa mRNA into FA cells *(14)*, transfecting total mouse genomic DNA *(13)*, and by studying non-human systems which carry defects analogous to FA such as with the Chinese hamster mutant V-H4 *(2, 44)*. However, none of these studies were able to uncover an FA gene. Recently though, the FA-C gene was cloned using a novel method of transferring a cDNA expression library using the Epstein-Barr virus as a vector, into a FA-C lymphoblastoid population and selecting out the plasmid which corrects the FA sensitivity to DNA cross-linking agents. This method, with minor variations, serves a the basis for the cloning of the FA-B gene *(52, 53)*.

The cloning strategy used by Strathdee et al. (1992) has a number of advantages to the other methods sited. The most important of which involves the use of the mini-Epstein-Barr virus (EBV) as a vector to insert cDNA into cells. The mini-EBV contains only the essential genes needed for its maintenance inside a cell line and is only a fraction of the size of the normal EBV (10-20 kb compared to approximately 170 kb), making it small enough to efficiently transfect into cells using standard methods of transfection. Unlike most plasmids, the mini-EBV (as well as the EBV) can be maintained as an episome inside mammalian cells as long as it is in the presence of the protein EBNA-1 and contains the origin of replication (oriP) driven by a promotor. Episomal plasmids can then be easily recovered using standard laboratory techniques*(52)* in contrast to linear plasmids which are integrated into the genome and thus are more difficult to pull out. Linear plasmids, when integrated, may also disrupt the expression of crucial genes required for the proper functioning of the cell, especially if it is randomly integrated. Upon integration, plasmids may also be rearranged or altered (53). Once retrieved, episomal plasmids can then be isolated and amplified by shuffling them into bacteria. The cDNA contained in the plasmids can be isolated by a simple enzyme digestion at the restriction sites flanking the cDNA sequence (see figure 3).

Approach

Choosing the cell line. Why FA-B.

At the start of the study, three complementation groups remained to be cloned, FA groups A, B and D, with group C being cloned earlier by Strathdee et al., 1992 *(53)*. The cloning of FA group D was rejected because it is the least sensitive of the four FA cell lines to cross-linking agents, making it difficult to differentiate when the cell line has been corrected and one of the only known FA-D cell lines, HSC 62, a lymphoblastoid cell line, has a doubling time of approximately twice that of the other FA cell lines (personal data).

FA group A was not totally ruled out though it is known that more than one other laboratory is pursuing the cloning of this gene. FA group B was then chosen based on these and other reasons, one of which is that, unlike FA-D cells, it has a cross-link sensitivity (based on the EC10)10-100 times greater than normal cells, depending on the agent used. This makes it easier, for selection purposes, to differentiate between corrected and uncorrected cells. It is, next to FA-C cells, the most sensitive of the remaining complementation groups (*personal data, 52, 53)*. FA-B cells were shown as well to have the highest rate of spontaneous and induced chromosomal aberrations than either FA-A or FA-D cells *(15, 16)*.

Obtaining a cDNA library.

There were two ways to acquire a complementary DNA (cDNA) library. The first was to construct one by collecting the total messenger RNA (mRNA) of a normal cell line and then synthesizing the complementary strand of each mRNA segment. In this way, the coding sequence for each gene that is expressed in the cell can be collected. These segments of cDNA can then be inserted in an mini-EBV vector, with each plasmid taking up one piece of cDNA. So in a large number of mini-EBV vectors, a whole cDNA library can be constructed. The other option was to purchase a cDNA library that was commercially manufactured. For the sake of time, the latter option was chosen.

Transfection Methods

Once a cDNA library, contained in an EBV shuttle vector, was obtained then the major task was to find an efficient method of transferring the

plasmid DNA into the cells. Enough cells must take-up the plasmid to insure that the entire library is expressed. The most common way to transfer plasmid DNA into cells is by transfection and in this project three different transfection methods were employed; electroporation, lipofection and endocytosis-fection.

Electroporation is a common method to fuse cells and to insert pieces of DNA into cells. Media containing cells and the DNA are placed into a small cuvette or chamber with a metal plate on two opposing sides. The chamber or cuvette is placed in an electric field, with a positive charge on one plate and negative on the other, where it receives an electrical current of a given voltage and capacitance for a short duration. During the passage of an electrical current, all negatively charged particles in the chamber will migrate to the positive charged plate. The speed of the migration depends on the mass of the particles with the smaller particles, such as the negatively charged plasmids, migrating at a much greater speed than the more massive cells. Thus the plasmid DNA "shoots" through the cells like a bullet, remaining in a certain portion.

Lipofection is a method of transfecting DNA by joining the negatively charged DNA with the cationic liposome mixture. The DNA-liposome complex fuses easily with mammalian cell membranes where it is taken up by the cell.

Endocytosis-fection is a relatively new system of transfection involving the association of DNA with adenovirus and the ligand transferrin, two components which have receptors on the membrane of most cells, and adding this complex to cells where it binds to receptors on the cell surface. Once attached to the membrane, the DNA complex is efficiently taken up by the cell via endocytosis. Inside the cell, a component of the adenovirus, thought to be the capsid protein, induces a drop in the pH which disrupts the endocytotic membrane, allowing any DNA associated with it to escape and sparing it from degradation by the endocytotic enzymes. The DNA is then free to migrate to the nucleus *(11, 12, 58)*.

This system has been found to be extremely efficient for transfecting DNA into mammalian cells, up to 100% in some cell lines *(11, 58)*. There has of yet been no reports of the successful use of this method to stably transfect plasmid DNA, possibly indicating a problem in doing so. Indeed this replication-defective adenovirus may be deleterious to the survival of

the cells either because it is not totally replication-defective or because it becomes replication-competent via recombination with endogenous virus's in the host cell. It has also been postulated that a component of the virus, the penton protein of the capsid, is toxic to mammalian cells *(9, 12)*.

Experimental Plan.

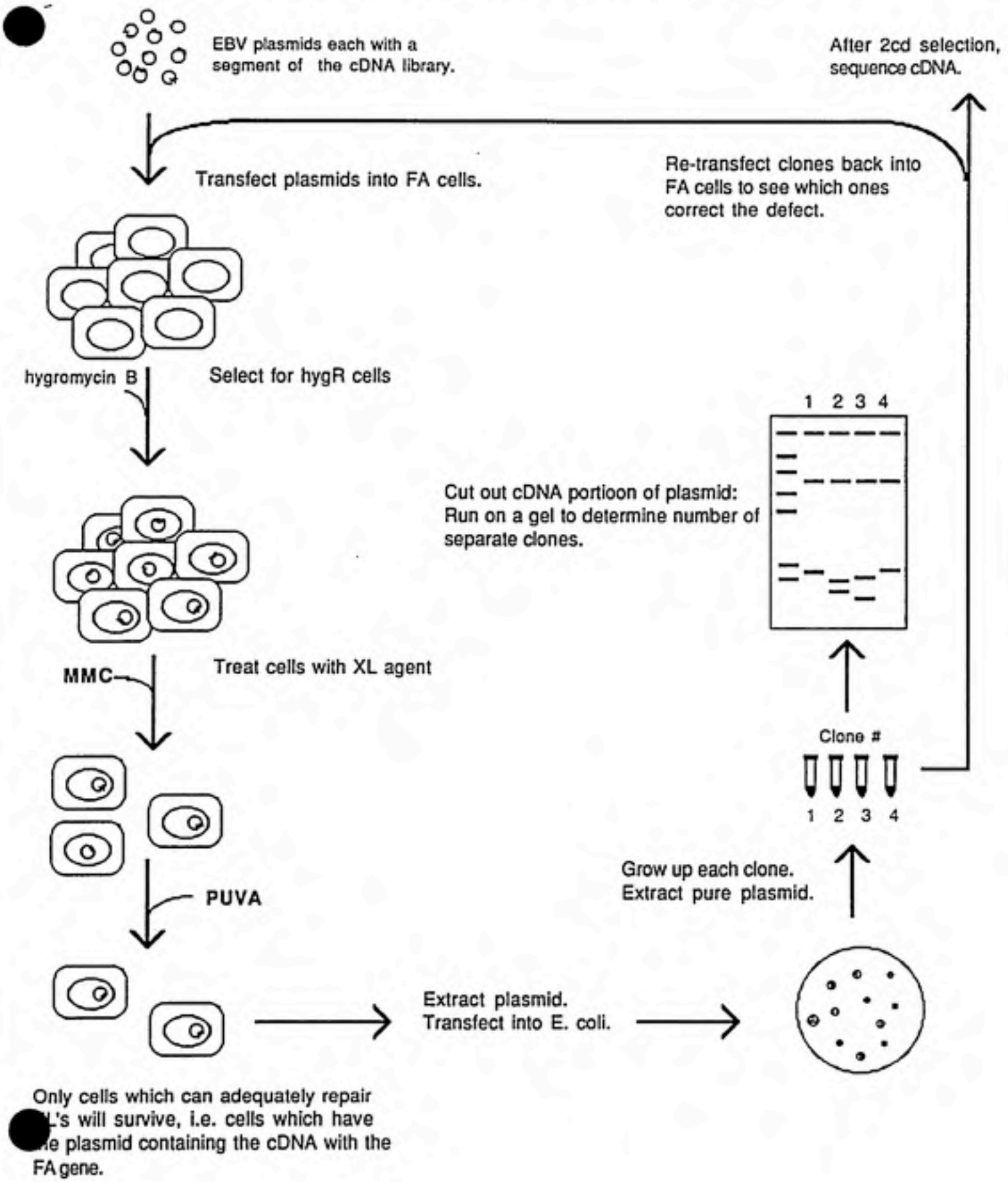
Once the best transfection method has been found and an EBV-cDNA shuttle vector and FA cell line been obtained, then an entire cDNA library must be inserted into an FA cell population. The number of genes in the human genome has been estimated to be 1.0×10^5. Therefor at least this many cells must be successfully transfected in order to insure that a majority of the library is expressed in the cells. To greatly increase the chances of having the entire library expressed and in high enough numbers to pull out the FA gene, at least ten times that amount or 10^6 clones should be obtained. Since the plasmid has a gene conferring hygromycin resistance, the cells which take-up the plasmid can be selected by treating with hygromycin B and pulling out the survivors. Once the survivors of the hygromycin treatment have been obtained then the corrected cells containing the FA gene can be selected for by treating the population of cells with first one and then another DNA cross-linking agent at doses which are lethal to the majority of FA cells but not to normal cells. Cells which survive treatments with both cross-linking agents will most likely contain a plasmid with the cDNA containsing the FA gene.

To confirm that the FA gene has been found, the plasmids from a large population of these cells must be extracted and then transfected into E. coli where each bacteria will take-up only one plasmid. Mini-plasmid preps can then be performed to obtain large numbers of each individual plasmid. Each separate plasmid can then be run on an agarose gel to determine its size and to ultimately determine how many distinct plasmids there are. The individual plasmids, starting with the ones recovered in the largest number can be introduced back into the FA cells and can be selected once again with the two DNA cross-linking agents. Plasmids conferring resistance to these agents after this second round of selection should contain at least some portion of the FA gene of interest (figure 3). Strathdee et al., found that in the pool of plasmids recovered form the two

cross-linking treatments, only a small number of them actually corrected the defect. The others were "passenger" plasmids that were maintained along with the correcting plasmids. This is due to the inherent stability of the EBV episomal plasmid in the presence of EBNA-1.

The final step would be to recover the cDNA in all the individual plasmids that conferred resistance by enzyme restriction, and sequencing them, noting consensus sequences contained in all of the plasmids.

FIGURE 3: THE CLONING SCHEME OF THE FA-B GENE.



Chapter III: MATERIALS AND METHODS

Cell lines and cell cultures.

The A9 mouse cell line with human chromosome #20 (HC20) tagged with neomycin resistant gene was obtained by Dr. R. Misra. HSC's 72, 99, 230, and 62, human, EBV transformed lymphoblastoid cell lines from FA groups A, A, B and D respectively, HSC 1199-11-2, a human SV40 transformed FA fibroblast, and HSC 92 and 93, normal human lymphoblastoid cell lines, were the generous gift of Dr. Manual Buchwald, (Hospital for Sick Children, Toronto, Canada). GM6914A, a human SV40 immortalized fibroblast, and GM1309B, a primary human fibroblast, officially classified as belonging to FA group A, were purchased through the Human Genetic Mutant Cell Repository (Camden, N.J.).

Media and Cell culture

The A9 mouse cell cultures were maintained in RPMI 1640, (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 ug/ml glutamine and 100 ug/ml penn-strep and maintained in 800 ug/ml G418 (Gibco). They were split 1:10 - 1:15 every 5-6 days.

The FA lymphoblast cell lines HSC 72, HSC 99 and HSC 230 were maintained in RPMI 1640 media and supplemented with 10% FBS or 10% bovine calf serum (BCS) as well as glutamine and penn-strep. They were split 1:8 every 5-6 days. The normal lymphoblast cell lines HSC's 92 and 93 were maintained in RPMI 1640 media supplemented with 10% FBS or BCS as well as glutamine and penn-strep and were split 1:10 every 5-6 days.

The transformed fibroblast cell line, GM6914A was maintained in Eagles minimal essential media (EMEM, Gibco) and supplemented with 10% FBS, glutamine and penn-strep. They were split 1:10 every 5-6 days. GM1309B was maintained in EMEM supplemented with 10% undialysed FBS with glutamine, penn-strep and non-essential amino acids and was split 1:5 every 5-6 days. The FA fibroblast cell lines, HSC1199-11-2 was maintained in EMEM media and supplemented with 10% FBS as well as glutamine and penn-strep. They were split 1:4 every 6-7 days.

Plasmids used:

The lacZ expression vector, pH200 was constructed by T. Sun of the Vos lab and is a mini-EBV plasmid containing the genes, *oriP*, the origin or replication in mammalian cells which contributes to the stable maintenance of the cell as an episome, *ori lyt*, the origin or replication in mammalian cells during the lytic cycle, hyg^r, hygromycin resistance gene for selection in human cells, and amp^r, the ampicillin resistance gene for selection in bacterial cells, all of which are driven by an *SV-40 promotor*. pH210 is the same construct as pH200 with the addition of *lacZ gene* driven by the SV40 promotor. pH250 is a similar construct as pH200 but with the addition of a CMV promotor and the lacZ gene (figure 4).

pDR2 is a commercial EBV plasmid containing a cDNA library (Clontech Inc.) derived from human placental cells. It contains an *oriP*, *Col E1 ori*, for replication in E. coli cells, *EBNA-1*, also for stable maintenance of the plasmid as an episome, hyg^r for selection in human cells, amp^r for selection in bacteria, a cDNA insert and an *RSV-LTR*, the Rous-Sarcoma virus promoter for the expression of the cDNA in mammalian cells, (figure 5).

METHODS: Project One:

Microcell-mediated chromosome transfer procedure.

The MMCT procedure is based on the protocols presented by Fournier, (1981), Saxon et al., (1985), and Sanford and Stubblefield, (1986) and Dr. B. Weisman (unpublished) and can be seen in figure 2 *(20, 45, 48)*. It can be broken down into four basic parts, consisting of the following:

1. Recipient and donor cell preparation.

Approximately 1.0 x 10^6 A9 cells were seeded into each of six T-25 Nunclon flasks four days prior to the day of fusion to be 80% confluent 2 days before the fusion. At this time colcemid was added at concentrations ranging from 0.005 - 0.2 ug/ml. 2.0 x 10^6 GM6914A cells or 3.0 x 10^6 GM1309B cells were seeded into a each of two 100 mm tissue culture plates (Falcon) 2 -3 days prior to the fusion so that they would be 80% confluent on the day of fusion. One dish received the MMCT treatment with the microcells while the other dish received the MMCT treatment with no microcells. FA lymphoblast were seeded at 2.0 x 10^6 cells per 100 mm dish 3 days prior to the fusion day so that they would be in log

FIGURE 4: MINI-EBV EXPRESSION VECTORS

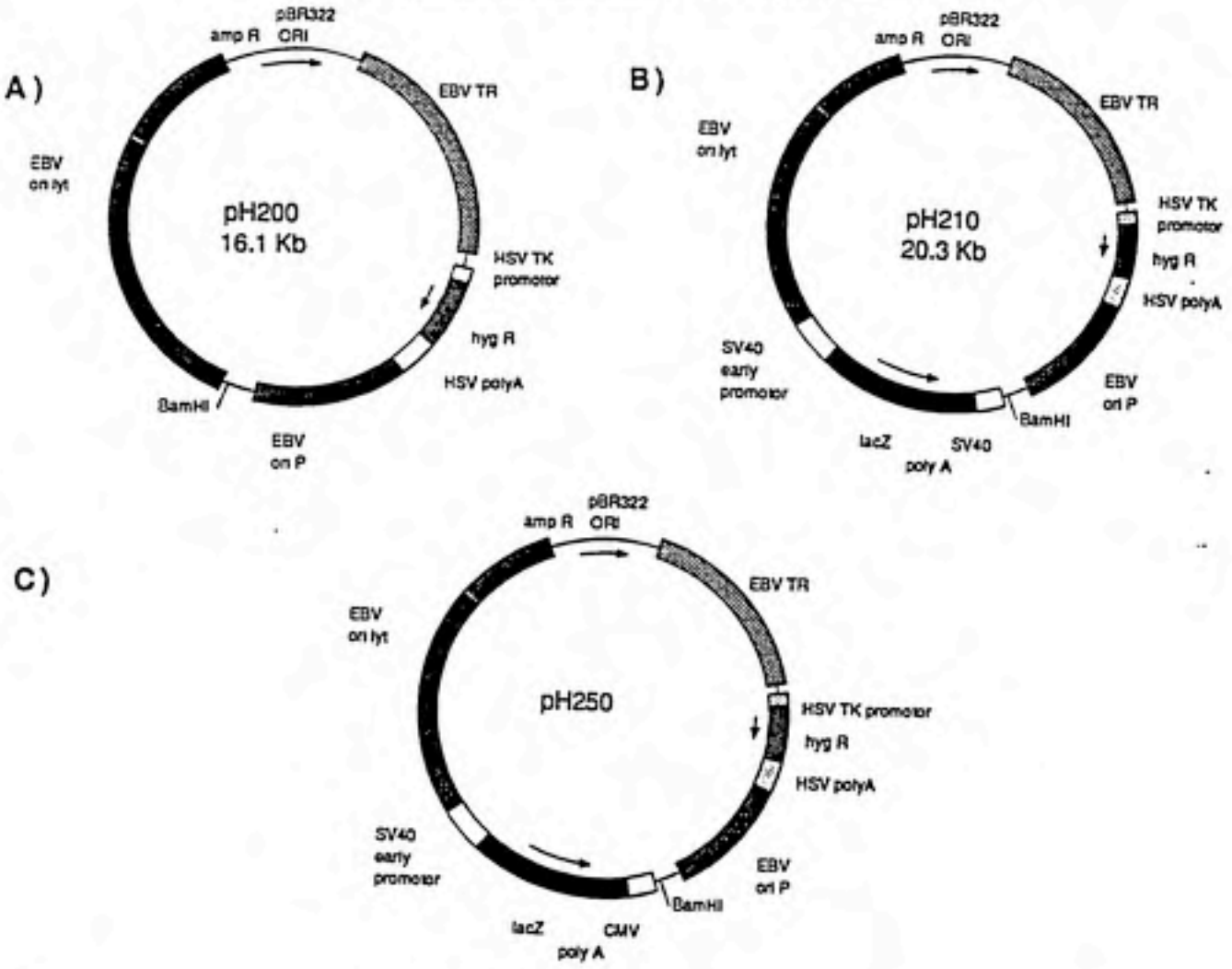


Figure 4: Three mini-EBV plasmids:" A) pH200, a non-lacZ containing mini-EBV plasmid, B) pH210, the same construct as pH200 except a SV40 driven lacZ gene has been inserted at the BamHI site, and C) pH250, the same construct as pH200 except a CMV driven lacZ gene has been inserted at the BamHI site. Genes of interest are: EBV oriP, the mammalian origin of replication, hyg R, the hygromycin resistance gene driven by HSV TK, the Herpes simplex virus thymine kinase promotor, and amp R, the ampicillin resistance gene.

FIGURE 5: pDR2 EBV-based cDNA library

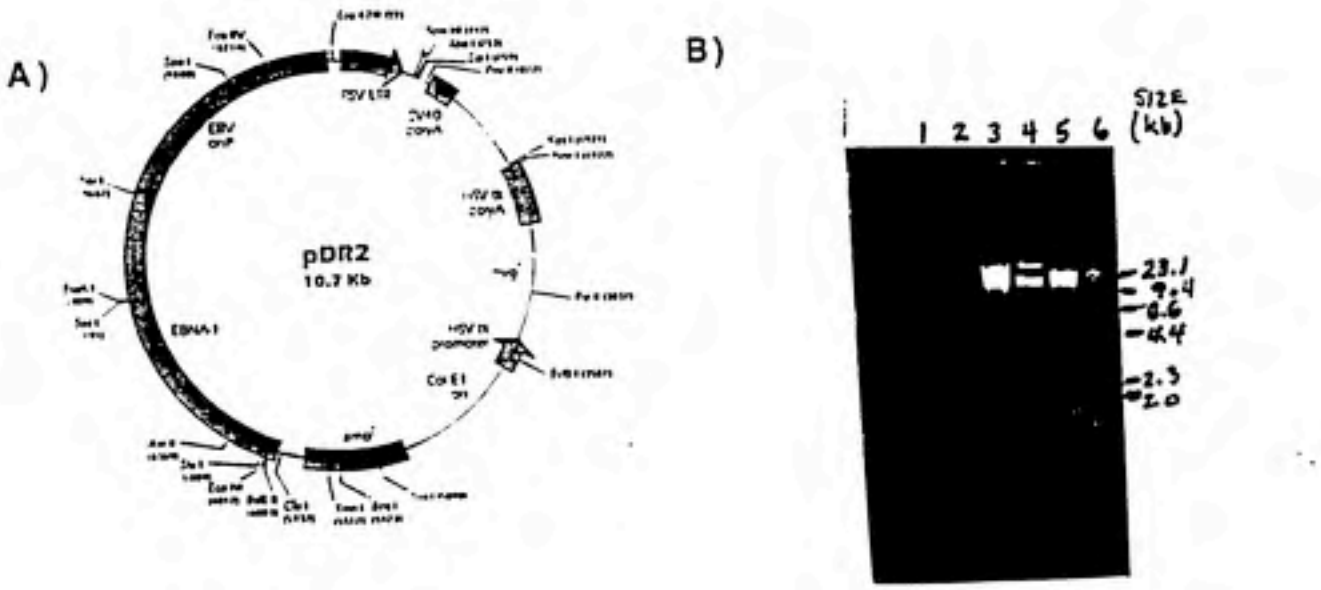


Figure 5: The pDR2 cDNA plasmid. A) Map of the pDR2 plasmid containing *oriP*, an origen of replication for mammalian cells, *Col E1 ori*, for replication in E. coli cells, *EBNA-1*, for stable maintenance of the plasmid as an episome, *hygr* for selection in human cells, *ampr* for selection in bacteria, a cDNA insert and an *RSV-LTR*, the Rous-Sarcoma virus promoter for the expression of the cDNA in mammalian cells, and B) a restriction digest of pDR2 plasmid with BamH1 and Xba enzymes, the two restriction sites flanking the cDNA segment. Lane 1 - plasmid alone, lane 2 - l-Hindi marker, lane 3- pDR2 digested with Xba and BamH1, lane 4 - pDR2 digested with Xba only, lane 5 - pDR2 digested with BamH1 only, lane 6 - l-Hindi marker. There is a smear of DNA in the lower portion of lane 3, indicating the presence of cDNA fragments of different sizes.

growth phase on the fusion day.

2. Isolation of the microcells.

On the day of the fusion the media from the T-25 flasks containing the A9 cells was removed and the flasks were filled with pre-warmed cytochalasin B (100 ug/ml). After 20-30 minutes at 37^0 C, the six flasks were placed, along with 100 ml of distilled deionized water (ddH_2O) into each well of a Sorvall SS-45 rotor and spun at 12,000 rpm's for 1 hour at 25-30^o C. When the flasks were are removed, a white pellet containing the microcells and cell debris, was apparent at the bottom corners. Under sterile conditions, the cytochalasin B was taken out, (and filtered through a 22μ filter to be used again), and 1.5 ml of fresh unsupplemented RPMI media (UM) was added to each flask. The pellets in the flask were resuspended in the media and collected in a 15.0 ml centrifuge tube. The media collected was then spun down at approximately 2100 rpm's for 10 minutes in an IEC tabletop centrifuge.

After spinning, the media was aspirated off and the pellet resuspended in 10 ml's fresh UM. It was then filtered through 8-μm, 5-μm and 3-μm polycarbonate filters (Nucleopore, Pleasonton, Ca.) serially and collected in a centrifuge tube. This purified microcell pellet suspension was then spun at 2100 rpm's for 15 minutes.

3. Fusion of the microcells and recipient cells.

At this point the fusion methods can vary depending on if the recipient cells are monolayer or suspension cells. Below are four variations employed: For monolayer recipient cell lines, GM6914A and GM1309B.

Standard protocol:

The monolayer recipient cell lines GM6914A or GM1309B were washed twice with PBS in preparation for the fusion. After the purified microcells were pelleted and the media was removed the pellet was then resuspended in 2.0 mls of PHA (100 ug/ml) and immediately added to one of the 2 plates. 2.0 mls of PHA (same concentration) with no microcells was added to the control plate. The dishes were then incubated for 5 minutes at RT and 5-15 minutes at 37^0 C, depending on the toxicity of the

cell line to the PHA. After a given time the PHA was taken off and 1.0 ml of 45 % PEG was added for one minute after which they were washed three time consecutively with UM. After the last washing, 10 mls of complete growth media was added to the cells which were then incubated at 37^0 C for at 24 -48 hours.

Modified protocol.

The same procedure was followed with the exception that the recipient cell line GM1309B was grown in 6-well plates and when the PHA-microcell suspension was added, they were centrifuged in an IEC centrifuge for 5 minutes at 1500 rpm's This variation was employed in an attempt to improve the chances that the microcells would attach to the cells.

Suspension recipient cells. Modified protocol #1.

Approximately 1.0 x 10^7 lymphoblastoid cells were placed into each of two 15.0 ml centrifuge tubes and spun down at 1200 rpm's for five minutes in a tabletop IEC centrifuge. After spinning, the media was removed and the cells were washed once with UM. After washing, the media was aspirated from both tubes. To the first tube, the microcell-PHA mixture was added and to the second tube, 2.0 mls of PHA with no microcells was added. The cells were exposed to PHA for approximately 5 minutes at RT and 10 minutes at 37^0C. After this time 2.0 mls of UM was added to inactivate the PHA and the cells were spun down at 1300 rpm's. The PHA was taken off and 2.0 mls of 45% PEG was added for one minute, after which time 10.0 mls of UM was added and the cells spun down at 1500 rpm's. This washing procedure was repeated 1-2 times. After the last time, the cells were resuspended in complete RPMI media with 10% FBS, placed into a 100 mm dish and incubated at 37^0C.

Modified lymphoblast protocol #2.

Approximately 2.0 x 10^7 lymphoblast cells in the log phase of growth were placed into a 50 ml centrifuge tube and spun down at 1200 rpm's for five minutes. After spinning, the media was removed and the cells were washed once with UM. After washing the media was removed and the cells were resuspended in 6.0 mls of fresh UM. Three mls of this solution, approximately 1.0 x 10^7 cells, were placed into each of two wells in a 6-

well plate (Falcon). The plates were spun down at 1500 rpm's for 10 minutes to obtain a forced monolayer. At this point the UM was removed from the cells and 2.0 mls of PHA-microcell suspension was carefully added to the cells and incubated the appropriate amount of time. After the appropriate time, the PHA was removed and 2.0 mls of 45% PEG was added. After one minute in PEG, three to four mls of UM was added to the wells and the cells were dislodged using a Pasteur pipette. This solution was then added to a 15 ml centrifuge tube containing 10 ml of UM and spun down at 1500 rpm's. This washing procedure was repeated 1-2 times. On the final time, the cells were resuspended in complete RPMI media with 10% FBS, placed into a 100 mm dish and incubated at 37^0C.

4. Selection of transformed cells.

Two days after the fusion, the cell were placed on selection in G418 (neomycin, [Fisher]) for up to 25 days, changing the media every 5-7 days. For the GM1309B and GM6914A fibroblasts, 200 and 800 ug/ml of G418 respectively were used. For the FA-A lymphoblast, 800 ug/ml G418 was used though other concentrations, of 400, 1000, and 1200 ug/ml were tried as well.

Project Two.

Plasmid Preparation: All of the plasmids were prepared by the methods described in Current Protocols for the Cesium Chloride-Ethidium Bromide preparation of plasmid DNA *(5)* with some minor alterations which include adding a LiCl precipitation step after the first ethanol precipitation, in order to remove part of the RNA, and placing the DNA through two rounds of CsCl separation.

The pDR2 plasmid came incorporated into a λ phage. AM1 E. coli were infected with approximately 1.0 $x10^9$ phage particles at which point the pDR2 insert was detached from the phage by the addition of ITPG which signals recombination to occur at the sites flanking the pDR2 plasmid. After this normal plasmid preparation protocol was followed.

Following the second round of CsCl centrifugation, the plasmid band was removed and TE saturated iso-propanol was added to remove the ethidium bromide. After this, the solution was dialysed for 2 days in a solution of 1X TE buffer (10 mM Tris, 1 mM EDTA). The buffer was changed three or more times during this time.

Quantifying plasmid: To determine the amount of plasmid, 20 ul of the plasmid solution was diluted into 1000 ul ddH_2O. Using a spectrophotometer, the OD at 260 and 280 nM was then taken. If the ratio of OD at 260 over 280 was 1.8 or over, the amount of plasmid could be determined by the following formula:

OD at 260 nm x dilution factor x 50 mg/ml = ug of plasmid/ml

To determine the purity and the size of the plasmid, approximately 5.0 ng of plasmid was placed in one lane of a 1% agarose gel and run for 30-45 minutes at 90 volts. The λ-hindI marker, which has bands of pre-determined size, was run in an adjoining lane. To correctly determine the size of the plasmid, the plasmid was cut with restriction enzymes and then run on the gel to confirm the presence of cDNA fragments in the pDR2 plasmid (figure 5-B).

Transfection methods:

Electroporation: 5.0×10^6 cells in log phase growth in 0.3 mls RPMI 1640 media with 10% FBS, was added, along with 20.0 ug plasmid DNA, to a BioRad 0.4 cm cuvette and placed on ice for 10 minutes. After this time the cuvette was placed in the BioRad Gene Pulser electroporation chamber and electroporated at the desired voltage (v) and capacitance (uF). After electroporation, the cells were placed back on ice for 10 minutes and then diluted in 10 mls of complete media and incubated at 37° C.

Lipofection: Method A: For each reaction: 40.0 ul of Lipofectin® and 15.0 ug of plasmid DNA were added, respectively, into each of two 3.0 ml polystyrene tubes containing 1.5 mls of OPTI-MEM media (Gibco). The contents of each tube were then mixed and allowed to incubate for 10 minutes at RT. It was then, along with 5.0×10^6 lymphoblastoid cells previously washed in PBS, added to one well of a 6-well plate (Falcon) and allowed to incubate at 37° C for 12-18 hours. The OPTI-MEM media was then removed and the cells were resuspended in complete RPMI media and placed back in the incubator at 37° C.

HSC's 72 and 230 were tested with this method to determine the short and long term transfection efficiency using lipofection. In one test, these

two cell lines were transfected with four different plasmid, pH200, pH210, pH250 and pDR2, all of which contain the gene for hygromycin resistance. After two days approximately 2.0 x 10^6 cells were used for the ONPG assay and the remainder were placed on 200 ug/ml hygromycin B.

Lipofection: Method B: For each reaction: Into two separate polystyrene tubes were placed, respectively, 6.0-12.0 ug of plasmid DNA in OPTI-MEM media to a volume of 100 ul and 20.0 - 40.0 ul of Lipofectin® with OPTI-MEM media to a volume of 100 ul. The contents of each tube were combined and allowed to incubate at RT for 10 minutes. After this time the DNA-liposome suspension was added to the cells, (pre-washed once in OPTI-MEM media), and resuspended in 800 ul of this same media. This solution was incubated for 7-14 hours at 37^0 C at which time 4.0 mls of media was added to the cells which were returned to the incubator for 24-36 hours.

This method was used to test the lipofection transfection efficiency of the FA-B lymphoblastoid cell line, HSC 230. Into each of four wells of two 6-well plates were placed 2.5 and 5.0 x 10^6 HSC 230 cells, respectively. Then to the four wells of each plate was added DNA-liposome complexes incubated, as described above, in ratios (ug DNA to ug Lipofectin®) of 6:12, 6:24, 12:24 and 12:36. The plates were incubated for 8 hours after which time 4.0 mls of complete media was added to each well. 48 hours later an ONPG assay was performed to determine the level of β-galactosidase expression.

Endocytosis-fection: A detailed description of the preparations of the materials used for this assay are described in Wagner et al., 1992, and Cotten et al., 1992 *(11, 58)*. The material used for this experiment was prepared and generously supplied by Dr. D. Curiel, (UNC-Ch School of Medicine).

DNA-Adenovirus conjugate preparation: (per reaction) On the day of the transfection, 0.25 x 10^{10} particles of replication-incompetent adenovirus (A249) was aliquoted into a 15.0 ml centrifuge tube. In a separate tube, 1.25 ug of monoclonal antibody-poly-lysine (MP301pL) was diluted in 125.0 ul of 50 mM HBS (150 mM NaCl/20 mM HEPES, pH 7.3), added to the adenovirus, and incubated at RT for 30 minutes. After this time, 6.0 ug of

plasmid DNA was diluted in 125.0 ul of HBS in a separate tube and added to the first tube and again incubated at RT for 30 minutes. Lastly 3.6 ug transferrin poly-lysine (hTFpL) and 2.6 ug poly-L-lysine 295 (pL295) were placed in 125.0 ul of HBS and again added to the first tube and allowed to incubate for 30 minutes (see figures 6 and 7).

At this point the DNA-adenovirus complex was added to the cells in 1.0 mls of media containing 2.0% FBS. After 1-2 hours at 37^0 C, media with FBS was added so that the cell concentration was 5.0 - 7.0 x 10^5 cell/ml and the FBS concentration was 10.0%. The cells were then incubated for 48 hours after which time they were placed on selection and/or the appropriate assays were performed.

Test Experiment #1: In the first trial, approximately 20 hours pre-transfection, 6.0 x 10^6 cells in 10.0 mls were seeded into each of two 100 mm plates. To one plate was added 50.0 ug/ml desferrin in order to induce formation of transferrin receptors on the cell surface. The other plate received no desferrin. The following day, 20% of the cells from each plate were taken and used for the endocytosis-fection experiment. (The remaining cells were used for a parallel transfection using electroporation.) At the time of transfection, in a 60 mm tissue culture dish (Falcon), 0.5 ml of the DNA-adenovirus-transferrin-poly-lysine complex was delivered to 1.5 x 10^6 actively dividing cells in 1.0 ml of media containing 2.0 % FBS, and allowed to incubate at 37^0C for 1.5 hours. After this time 1.5 ml of complete media with 18% FBS was added to each plate which were placed in a 37^0 C incubator. After thirty hours, the cells were counted and an ONPG assay was performed.

Test Experiment #2 : In the second experiment, the standard procedure, as described above, was used for four different trials, with variations in the amount of various components. 2.0 ug of the reporter plasmid pH210 was used instead of the 6.0 ug of the CMV-lacZ construct used in Test #1 in all four trials. This difference aside, trial 3 was done under the same conditions as in experiment 1. In trials 1, 2, and 4, no pL_{295} was used and as well, in trial 4, only 1.25 x 10^{10} particles, 50% of the normal amount, of adenovirus was used.

After the formation of the DNA-adenovirus conjugate, 0.5 mls of it was added to 4.0 x 10^6 HSC 230 cells in 1.0 mls of 2.0 % media. After 2 hours,

Figure 4: Construction of Adenovirus-DNA complex.

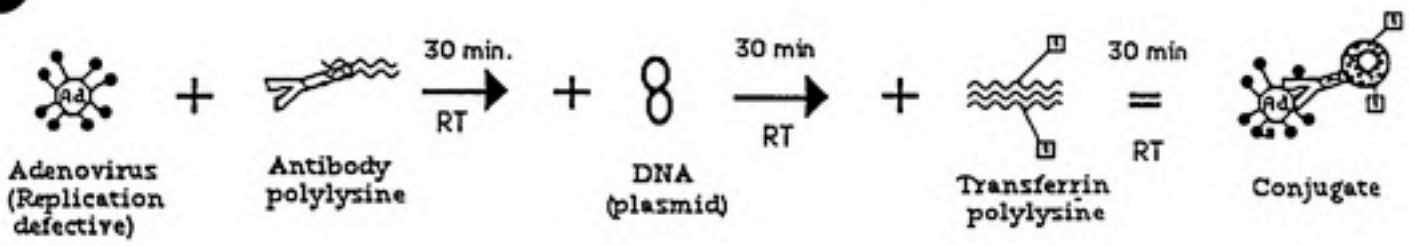
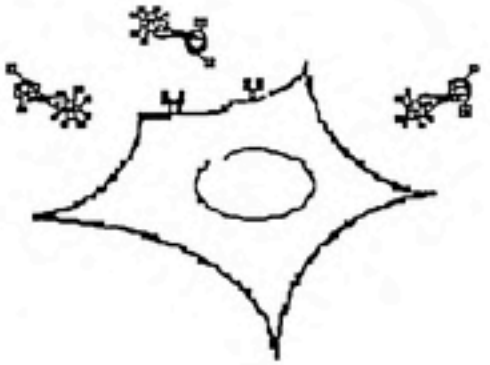
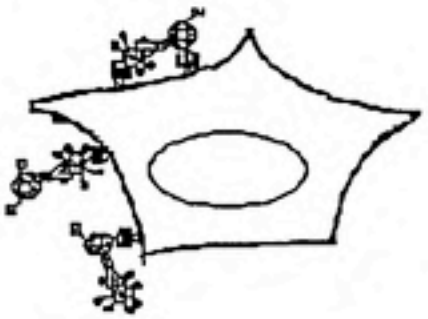


Figure 6: Procedure for preparing the adenoviral-DNA complex. The linker antibody-polylysine macromolecule is incubated with the replication-defective adenovirus providing a site for the plasmid DNA to attach. The transferrin-polylysine (hTFpL) then binds to the DNA causing it to fold into a "doughnut" shape, (11,12). The conjugate can then be delivered to the cells.

Figure 7: Mechanisms of the endocytosis-fection method of transfection.

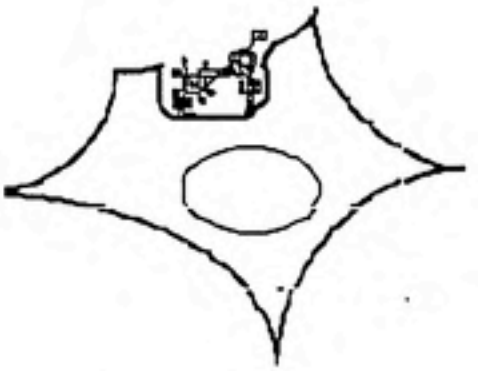
1. Adenovirus-plasmid conjugate is added to cells.

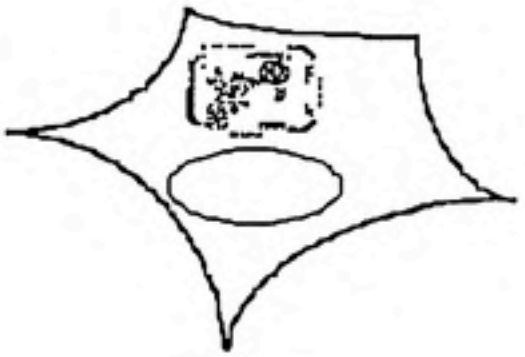




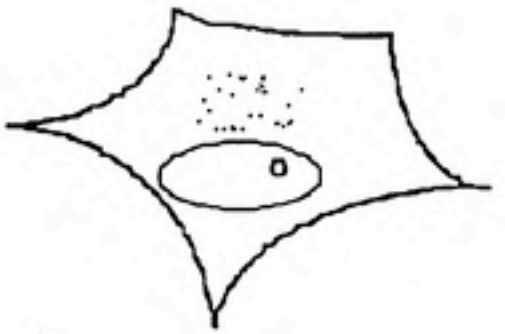
2. Conjugate binds to adenovirus or transferrin receptors.

3. Bound conjugate is taken up through endocytosis.





4. Adenovirus disrupts endocytotic membrane, allowing plasmid to escape.



5. Plasmid migrates to the nucleus.

4.0 mls of 10% FBS media was added. 48 hours later, the cells in each trial were counted and 1.0 x 10^6 cells from each trial was used for the ONPG assay. The remainder of the cells, at a concentration of approximately 4.0 - 5.0 x 10^5 cell/ml, were placed on 125 ug/ml hygromycin B. The cells were counted and the media changed every 4-5 days. After the tenth day the dead cells were removed by centrifugation on a ficoll gradient.

Determining Transfection Efficiency

In order to determine the most efficient method of transferring the plasmid into FA cells, plasmids containing the lacZ gene (which codes for the enzyme β-galactosidase) were transfected into FA cells. This enzyme breaks down the substrate ONPG (o-nitrophenyl-β-D-galactopyranoside) forming a yellow by-product, and subsequently the relative amount of the enzyme produced can be determined by measuring the optical density (OD) of the solution; the intensity of yellow being directly proportional to the amount of β-galactosidase. Two plasmids were used which contained the lacZ gene, one of which has the SV40 promoter (pH210) and the other which has the CMV promoter (pH250), the latter of which has been shown to be five times as strong as the former (T. Sun, unpublished data).

β-galactosidase or ONPG Assay.

A description of the β-galactosidase assay is described in detail by Lim and Chae, 1989 *(38)*. Briefly, from 1.0 - 2.0 x 10^6 cells were washed once with phosphate buffered saline solution (PBS) and resuspended in 0.5 mls of Hanks balanced saline solution (HBS). This cell solution was then added to a 1.5 ml eppendorf tube containing 0.5 ml of a 2X ONPG solution (7.0 mM o-nitrophenyl-β-D-galactopyranoside [ONPG] and 10% Nonidet-40 [NP-40] in HBS). This solution was mixed thoroughly and allowed to incubate at 37^0 C for various time points from 1-62 hours. At any time point, the solution was spun in an microfuge at 1350 rpm's for 5.0 minutes to pellet the cells and cell debris. The optical density (OD) at 420 nm of the supernatant was then measured in a spectrometer, using a plastic cuvette. Afterwards the cell solutions were retained and re-incubated.

To find the portion of the OD reading due to the presence of the transfected plasmid, the OD reading from cells transfected with a non-lacZ plasmid or no plasmid (the background level) is subtracted from the OD

reading of the lacZ-transfected cell line. This equals the corrected OD reading.

Corrected OD reading = OD at 420 nm of lacZ transfected cells - OD at 420 nm of non-lacZ transfected cells

To take into account the different numbers of cells sometimes used for the ONPG assay, the OD readings were normalized by dividing the OD reading by the number of cells used for the ONPG assay, to give an absorbance unit per cell.

$$\text{Absorbance unit per cell, (AU)} = \frac{\text{Corrected OD for cells transfected with lacZ plasmid}}{\text{\# of cells used in ONPG assay}}$$

Determining transfection percentage:

When the ONPG assay is performed on cells which have been transfected with a pH210, it was sometimes performed on a similar number of cells from a HSC 93p588Z, a normal lymphoblastoid cell line which carries the lacZ plasmid driven by the same promotor. After 3 hours, the OD was taken of experimental and control cells and an estimate of the percentage of cells transfected was calculated as following:

$$\text{Transfection efficiency, (\%)} = \frac{\text{Corrected OD reading of transfected cells}}{\text{Corrected OD reading of cells with lacZ plasmid}} \times 100$$

SENSITIVITY ANALYSIS OF SELECTING AGENTS

Hygromycin sensitivity:

HSC 230 cells were exposed to varying doses of hygromycin B to test their sensitivity. In the first trial, 5.0×10^5 cells/ml in 10.0 mls were added to each of six 100 mm dishes. 0, 50, 100, 150, 200 and 300 ug/ml hygromycin B were added to each of the dishes and the cell counts were taken every two days. After 6 days the growth percentage was calculated.

In the second trial, 0, 50, 100, 150 and 200 ug/ml hygromycin B, respectively, were added to each of two wells of a six well plate, each containing 6.0×10^5 cells in 3.0 mls. Cells were incubated six days at 37°C and then counted to determine the percent cell growth.

MMC sensitivity:

For each cell line, 1.0 x 10^5 cells in 1.0 ml were placed into each well of a 24-well plate. Each of the 6 columns of the plate were treated with a different concentration of MMC including 500, 100, 50, 10, 1, and 0 nM. The cells were incubated at 37^0 C for 5 days after which time the number of cell in each well was calculated and the percent cell growth was determined. This was performed on FA cell lines HSC's 230, 72, 62 and normal cell lines UC and HSC 93.

PUVA - Psoralen and UVA light.

2.0 x 10^5 cells/ml in 1.0 ml were placed into each well of a 24 well plate. Each of the 6 columns were treated with a different concentration of the psoralen derivative, HMT, from 0.1, 0.05, 0.001, 0.005, 0.001 and 0.00 ug/ml. After the HMT was added, the cells were incubated in the dark at 37^0 C for 10 minutes and then placed under 360 nm UVA light for 5 minutes. After this time the plates were placed back in the 37^0 incubator for five days at which point the number of cell in each well was determined and the percent cell growth was calculated.

The percent cell growth was determined by the following formula for treatment of any given cell line with an growth inhibiting agent:

$$\% \text{ growth} = \frac{(\text{Original cell \#} - \text{Day X cell number}) \text{ concentration Y}}{(\text{Original cell \#} - \text{Day X cell number}) \text{ no treatment}}$$

Where X is # of days after the treatment with chemical and Y is the concentration of that chemical administered to the cells

The Selection process:

Cells transfected with a plasmid containing the hygromycin resistant gene were selected in hygromycin at a concentration that inhibited growth 90-99% after 5-6 days. Cells were placed in the desired concentration of the selecting agent and every 4-5 days a cell viability assay was performed to determine the number of live and dead cells. After this the cells were either centrifuged and resuspended in fresh media with hygromycin or they were placed on a ficoll cushion (Accurate Chemical Co.) to remove the dead cell and then resuspended in fresh media with

hygromycin. Hygromycin-resistant colonies were seen after 3-4 weeks of selection.

Removing Dead Cells: The old media on the cells to be placed on ficoll was removed and the cells resuspended in 3.0 - 5.0 mls of fresh media. This suspension was then carefully placed on top of 3.0 mls of Lymphoprep® (Accurate Chemical Co.), a ficoll gradient, and spun for 10 minutes at 1250 rpm's and 10 minutes at 2000 rpm's. Live cells collect at the media-ficoll interface and dead cells pellet to the bottom. The live cells are then collected and washed once in 10 mls media to remove the ficoll. After washing, the cells are resuspended in 10.0 mls complete media along with the selecting agent and placed back in the incubator.

Cell Viability Assay: The number of living and dead cells in a culture was determined by treating a small volume of the cells with Trypan blue. Briefly, 100 ul of Trypan blue was added to the same volume of cell suspension, for a 1:1 dilution. Approximately 25.0 ul of this solution was added to a hemocytometer and the cells counted. The dead cell are stained blue while the living cells remain bright.

Chapter IV: RESULTS AND DISCUSSION

Experimental results-Project One:

At least 10 MMCT trials were attempted during an eight month period with no successful insertion of the HC 20. It was tried primarily on the FA-A lymphoblasts though it was also attempted on two FA-A fibroblast cell lines.

Table 1 shows an overview of the conditions used for most of the MMCT experiments. In one of the trials (#3) performed on GM6914A, neomycin resistant colonies were obtained, but this, upon later macroscopic and cytologic examination, appeared to be the result of a whole, not a micro-cell fusion. After two weeks, the cells began to take on the appearance of the original A9 mouse cell line and cytologic examination revealed that the mitotic spreads consisted mostly of mouse chromosomes. This result was most likely the result of a leak in the filter which allowed whole nuclei and cells through.

In two of the first trials, HSC 72 was used as the recipient cell line. The colcemid concentration used on the A9 cells was 0.002 ug/ml, a value one to two orders of magnitude lower than the normal values used in the literature. A microcell pellet was observed after the microcell purification in some of these trials, though it is likely that microcell concentration was small. The cells in the first trial were spun down in a 6 well plate to force a monolayer while the other was carried out in a centrifuge tube. The cells were placed under selection with 800 ug/ml of G418 and two-three weeks later they all had died. Since some microcell pellet was seen after the purification step in experiment 1, the colcemid concentration was not suspected as a problem. In the second experiment, the number of A9 cells treated with colcemid was doubled and the exposure to PHA was lessened. Though the experiment was not successful, the length of time the cells survived in G418 was an indication that the concentration was not too high. A primary reason for the failure of the experiment may be the low concentration of colcemid used to form the microcells though there are also

Table 1: Overview of MMCT experiments.

Exp. #	Cell line	Cell Type	Colcemid (ug/ml)	Method	G418 (ug/ml)	Resistant colonies?	Results:
1	HSC 72	lymphoblast	0.002	forced monolayer	800	no	cells were dead after 2-3 weeks.
2	HSC 72	lymphoblast	0.002	cent. tube	800	no	cells were dead after 10-14 days.
3	GM6914A	fibroblast	0.002	standard	800	yes	false positive: leak in filter.
4	HSC 99	lymphoblast	0.002	centrifuge tube	800	no	cells were dead after 10-14 days.
5	GM1309	fibroblast	0.05-0.2	standard	1000	no	cells looked sick after treatment.
6	HSC 99	lymphoblast	0.05-0.2	forced monolayer	800	no	cells were dead after 10-14 days.
7	GM1309	fibroblast	0.05	spin cells	200	no	cells looked sick after the treatment: died 4-5 days

The MMCT procedure is described in the Mterials and Methods. The reactions involving lymphoblast cells were carried out a.) in a six well plate where they were forced via centrifugation into a monolayer, or b) in a centrifuge tube. Cells were placed on G418 1-2 days after the experiment.

indications that the problem may lie with the nature of the cell line and cell type.

At a later time, a fusion experiment was carried out on another FA-A lymphoblastoid cell line, HSC 99. In this trial, the A9 cells were treated with a colcemid concentration of 0.05 ug/ml and as a result, a more noticeable microcell pellet was observed. The recipient cells were forced to a monolayer in a 6 well plate though when the microcell-PHA solution was added, the cells lifted up. After a 17 minute incubation in PHA, the cells were transferred to a centrifuge tube and spun down. After the PHA was taken off and the PEG added, the cells were diluted in UM and washed two consecutive times with UM and then placed in fresh media. They were placed on 800 ug/ml G418 two days later and typically, 10-14 days later, no live cells remained in either the control or experimental plate.

Two experiments were done on the primary fibroblast GM1309B. In both experiments, the colcemid concentration used to treat the A9 cells was 0.05-0.2 ug/ml and noticeable purified microcell pellets were seen. In the first experiment, over one-third of the cells lifted off after the PEG treatment, indicating that the treatment with PHA and PEG was toxic to the cells. Two days later the cells were split and placed on 1000 ug/ml concentration along with the PHA and PEG treatment, proved to toxic for the cells. No live cells were seen after 1-2 days. In the other experiment, the cells were spun in a centrifuge after the addition of the microcell and PHA. This was done to increase the chances the number of microcells that attached to the fibroblasts. Though the PHA and PEG times were lowered, the spin in the centrifuge as well as the PHA and PEG treatments once again damaged the cells and very few live cells remained 4-5 days after the experiment even thought the selecting concentration of G418 was lowered to 200 ug/ml.

Discussion

The FA-A lymphoblasts, represented by HSC's 72 and 99, were the cell line of choice for the majority of the MMCT experiments for a number of reasons. First, by nature of their cell type, lymphoblastoid cells can be grown in large numbers in relatively small volumes compared to fibroblast cell lines. This means a greater number of cells can be transfected for a given volume of media. A second reason is that these cell lines are

immortalized and they have a normal complement (2n=46) of chromosomes, unlike either of the fibroblast cell lines. GM6914A cells are immortal but have a rearranged karyotype and an average chromosome number of greater than seventy which is a major reason why more attempts were not made with them. GM1309B cells have a normal karyotype but they are not immortal. It was important to have an immortalized cell line because these generally grow faster and are more receptive to the uptake of foreign DNA than primary or unimmortalized cell lines *(27)*. Primary cells lines have a finite life span of undetermined length so may be unsuitable for an experiment which could potentially take many months to complete.

Also, a normal complement of chromosomes was deemed an important characteristic because of the ease and accuracy with which chromosome breaks can be counted and the location of the breaks identified. In a rearranged karyotype with a large number of chromosomes, such as occurs in GM6914A, all but chromatid breaks can be easily identified, with most double strand breaks and translocations being indistinguishable from the background chromosomes. In light of the fact that chromosome breaks can be easily induced in FA cells, and whose correction could easily be tested, the desire to count and identify spontaneous break points appears to be a secondary concern.

There are many disadvantages to using lymphoblastoid cells, most of which came to light after work with the subsequent project. One disadvantage is that modifications had to be made in the MMCT protocol to accommodate the use of suspension cells over monolayers. One of these is that when solutions such as media, PHA and PEG were to be removed from the cells, the cells had to be spun down to a pellet before the solutions could be taken off. This made it difficult, especially with PEG exposure, to expose the cells to an agent for a specific, short period of time because the exposure always included time in the centrifuge, even if the agent was diluted. This also contributed to a loss of cells which invariably occurred with each spin.

Additionally, it has come to light through the work in the second project that lymphoblastoid cells, especially FA lymphoblasts, are, in general, harder to transfect and select for resistance cells than are fibroblasts (personal observations). In transfection experiments on HSC 72,

efficiencies of no more than 1-5% have been gained using 5.0 - 15.0 ug's (2.0 - 7.0 x 10^{11} particles) of plasmid DNA (54). In MMCT tests performed on various human cell lines, no more than 35.0% of cells treated with colcemid formed microcells with the average being near 15.0% *(45)*. If the same holds true for A9 cells, then up to 1.0 x 10^7 out of the approximate 2.0 - 3.0 x 10^7 cells produce microcells with only a portion of these actually attaching to the cells and an even smaller portion being taken up. With this in mind, it is unlikely that a successful microcell fusion could be obtained with the FA lymphoblasts. This could be one reason why trial #5 using HSC 99 was unsuccessful. In this experiment, the appropriate amount of colcemid was used, a microcell pellet was observed after the purification step, the PHA and PEG toxicity was not a factor and the cells were selected on 800 ug/ml G418, dying off 10-14 days later.

In addition to having a low efficiency of transfection, the process of selection is more difficult using lymphoblasts (especially FA lymphoblasts) than fibroblast, once again based on observations made in the second project. Whereas in theory, one fibroblast cell is capable of propagating into a colony, lymphoblast cells must be at a certain threshold cell density before they will multiply to any degree (personal observation). Therefor a much higher percentage of lymphoblast than fibroblasts would have to be transfected in order for resistant colonies to appear. Also, once a fibroblast cell has died, it will lift off the plate, making it easy to distinguish and separate the live from the dead cells. With lymphoblast cells, the dead cells remain in the media with the live cells so it is more difficult to differentiate the two. The accumulation of dead cells is also thought to have a negative impact on the growth of the live cells. Therefor in this experiment, even if some cells did take up the neo^r tagged HC 20, it is questionable if they would be able to grow-up into large enough numbers to be detected. In light of these factors it would have been wise to focus greater attention on the FA-A fibroblasts.

CHAPTER V: CONCLUSIONS AND RECOMMENDATIONS

The microcell-mediated chromosome transfer is a common procedure used widely to transfer human chromosome into either human or rodent backgrounds. It has been used to establish a library of single human chromosomes in rodent cells backgrounds as well as to transfer individual human chromosomes to test for the complementation and correction of various genes *(48, 59)*. In the field of DNA repair disorders, the presence of the Ataxia telangiectasia group D (AT-D) gene was confirmed to be on HC 11 by the successful insertion of a HC 11 into AT-D cells by way of MMCT *(33)*. However, when the same procedure, using HC 20, was attempted on the Fanconi's anemia group A (FA-A) cells, a successful insertion of HC 20 could not be achieved. There are a few reasons which might account for this. For the FA-A lymphoblast cells, the problem most likely involves the nature of the cell line and cell type as described above. For the FA-A fibroblasts, this failure is due to a poorly planned and executed experiments. The GM1309B cells proved more sensitive to the procedure and selecting agent than had been anticipated. Only one trial was done on the immortalized fibroblast GM6914A cells which resulted in a whole cell fusion. In light of what is known now, concerning the nature of fibroblasts and lymphoblasts, more attention should have been focused on this latter cell line. It must be noted though that, concurrent to the time this research was being conducted, Dr. Margaret Zdzienicka of the State University of Leiden, The Netherlands, was also conducting similar MMCT experiments using the GM6914A cell line. In five separate trials she was also unable to successfully transfer HC 20 into this cell line, (Dr. M. Zdzienicka, personal communication). This fact underscores the difficulty of the introduction of foreign DNA into FA-A cells.

Improvement that could have been made.

There are a couple of improvements that if made, might have improved the chances for success. The first of which is to have done a detailed sensitivity analysis on the FA cell lines, especially the fibroblasts,

to the agents neomycin, PHA and PEG. This would have maximized the chances of achieving a successful selection by insuring that the cells would not be exposed to chemical concentrations which were too toxic for the cells to handle. Another improvement would have been to perform a MMCT on a normal cell line or a cell line in which a successful MMCT had been previously been performed. A positive transfer of a human chromosome into a normal cell line and not in an FA cell line using the same method would support the notion that failure in the FA cell line was due to the nature of the cell and not the procedure.

Recommendation for future research.

The quest to find all four of the FA genes is moving along at a rapid pace with a number of groups working on cloning each of the groups, with FA-group C having already been cloned. Therefor, it may be of little worth spending more time trying to locate the chromosomal location of the FA-A gene when its precise genetic location as well as gene code may be close to being discovered. If research were to be taken up again on this project, it would best to advisable to find an immortalized FA-A fibroblast cell line. Recently, the classification of GM1309B and hence the GM6914A cell line as belonging FA group A has been called into question by the discovery of a mutation in this cell line at the FA-C gene locus *(61)*. However, the authors remain doubtful that this represents a true FA-C mutation.

CHAPTER VI: RESULTS AND DISCUSSION-PROJECT TWO

FA-A and FA-B lymphoblasts were transfected with plasmids which contain the lacZ gene in order to determine the efficiency of transfection. Two days post-transfection they were placed in an ONPG solution and the OD was subsequently measured. To normalize the results between experiments, the OD was divided by the number of cells used for the assay to give an absorbance unit per cell or AU. They were also transfected with the cDNA library to see if stable maintenance of the plasmid could be obtained. The most common AU values obtained were in the range of 0.15 -1.0 (x 10^{-7}) for the SV40-driven plasmid though higher values were obtained with endocytosis-fection. These values, based on the second of the endocytosis-fection experiments roughly correspond to transfection efficiencies of 1.0-5.0%. In a FA-A lymphoblasts the cDNA-containing plasmid was able to be stably maintained with an AU, after correcting for plasmid strength, of only 0.15. (Since the CMV-lacZ is approximately five times stronger than the SV40-lacZ then values obtained with the former plasmid are divided by five to give a normalized result). However, for the FA-B lymphoblasts values two-three times higher than this failed to yield resistant colonies. The threshold value which will result in stable maintenance of the library is unknown.

Results of Transfections-Method I: Electroporation

Transient assays

The major factors influencing the outcome of transfection using electroporation are voltage and capacitance. Other important factors are cell volume, cell density, plasmid amount, cuvette size, and temperature. Numerous electroporation experiments were done on HSC 230 to determine the transfection level, all of which yielded relatively poor results.

In the initial experiment, D/R Raji cells, HSC 1199-11-2(monolayers), and HSC 230 (suspension) were electroporated with the pH210 plasmid under three different voltage (v)/capacitance (uF) conditions; 200v/ 960 uF, 150v/ 960 uF, and 200v/ 500 uF. All other factors were kept constant,

Table 2: Relative lacZ expression in FA-B cells transfected via electroporation

A) Original OD readings at 420 nm.

	ELECTROPORATION CONDITIONS		
Cell line	200/960†	150/960	200/500
D/R Raji	0.126	0.765	0.785
HSC230	0.118	0.454	0.369
HSC1199-11-2	0.027	0.078	0.050

B) Estimated AU (x 10^-7)

	ELECTROPORATION CONDITIONS		
Cell line	200/960†	150/960	200/500
D/R Raji	0.190	0.665	0.761
HSC230	0.170	0.354	0.299
HSC1199-11-2	0.018	0.076	0.018

All cells were transfected with the pH210 plasmid using standard electroporation protocol while varying the voltage and capacitance. Table 1A contains the original OD readings taken after 24 hours incubation in ONPG. Table 1B contains the estimated relative lacZ expression obtained by first subtracting the estimated background signal from the original OD readings then dividing this number by the estimated cell number used for the ONPG assay. The background signals and specific cell numbers were not taken and so estimates, based on subsequent electroporation experiments, were used. The numbers in 1B represent OD units per cell. D/R Raji is a hybrid cell line grown in a monolayer which had been successfully transfected via electroporation (52). HSC's 230 and 1199-11-2 are transformed lymphoblast and fibroblasts respectively, belonging to FA group B.

† = voltage (v)/ capacitance (uF)

including a cell density of 5.0 x 10^6 in a volume of 0.3 mls using 20.0 ug pH210 plasmid, in a 0.4 cm cuvette at 0.0°C. All of the cells were used for the ONPG assay 2 days after the transfection and the OD reading taken after 24 hours (table 2). The initial ONPG results seemed promising, with the solution of cells in ONPG turning noticeably yellow for some of the conditions. These corresponded to OD readings of almost 0.50 for the HSC 230 cells and over 0.75 for the D/R Raji cells. However taken in light of the high number of cell most probably used for the ONPG assay, the readings weren't as high as they appeared. Since the cell numbers were not counted, accurate AU value's could not be obtained especially for the first condition which had the highest voltage and the highest rate of cell death. However, for the latter two conditions, fairly accurate estimates could be made of the cell numbers based on subsequent electroporation experiments.

The results indicate that of the conditions used, the 150/960 and 200/500 voltage to capacitance conditions were similar in effect and were superior to the 200v/960 uF condition. However, as indicated, there is uncertainty as to the level of expression of the latter condition.

These results also suggest that FA-B lymphoblasts can be electroporated at a two-fold higher efficiency than the FA-B fibroblasts. The signals for the fibroblast were lower than the FA-B lymphoblast in all three conditions barely reaching above the background. They appear also to have an order of magnitude lower efficiency of transfection than the D/R Raji cells. Indeed the FA-B fibroblast grows at a slower rate and appear to be more sensitive to such factors as media pH than is either D/R Raji cells or HSC 230 (personal observation). Thus they may also have been more sensitive to the effects of electroporation.

Even with the error involved, the highest relative signals (AU's) were well below 1.0 x 10^{-7} for the FA-B cells and were slightly under 1.0 x 10^{-7} for the D/R Raji cells, indicating a low efficiency of transfection.

In a later experiment, lymphoblasts from three FA cell lines, HSC's 72, (FA-A), 230 (FA-B), and 62 (FA-D) were electroporated with the pH250 plasmid using standard conditions including 150v and 960 uF, conditions determined in the first experiment to be the most efficient (table 3, figure 8). The FA-B lymphoblast had a level of transfection between that of the FA-A and FA-D lymphoblasts. The estimated AU for HSC 230 was 0.8 x

Table 3: LacZ gene expression in FA lymphoblasts transfected by electroporation

Cell Line	Corrected OD (420 nm)	Est. cell # used in assay	Est. AU *
HSC 72	0.27	19.50	1.40
HSC 230	0.15	17.40	0.87
HSC 62	0.01	14.00	0.06

HSC's 72, 230 and 62 representing FA groups A, B, and D respectively were electroporated under standard conditions of 150 volts, 960 uF capacitance with 5 x 10^6 cells, using the pH250 plasmid. The ONPG assay was performed 48 hours later. The corrected OD = OD (lacZ transfected cells) - OD (non-lacZ transfected cells). All cell numbers expressed in units x 10^5 and are estimated based on subsequent experiments.
* = absorbance unit per cell x 10^-7.

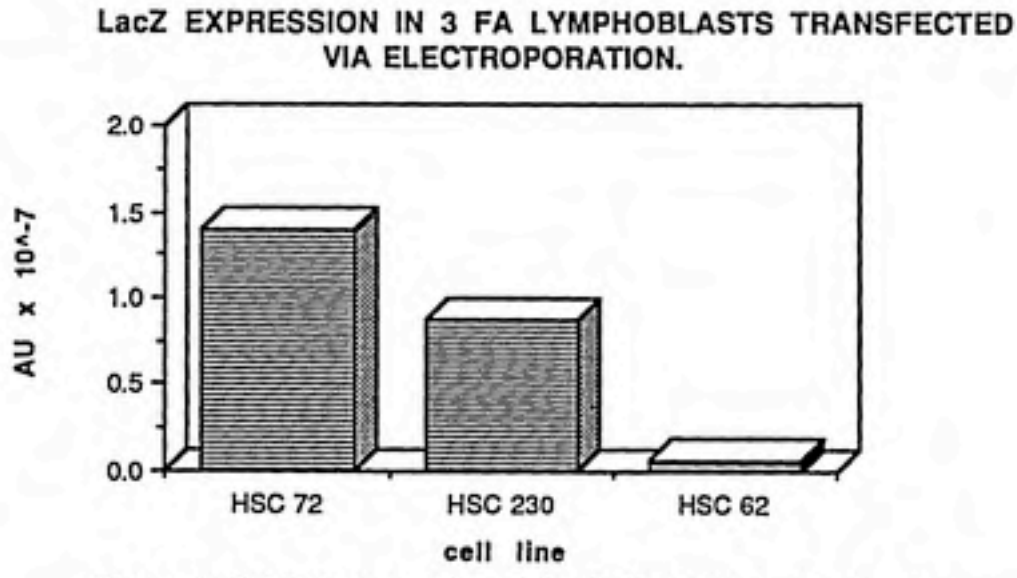


Figure 8: Relative expression of the lacZ reporter gene in three FA cell lines transfected with plasmid pH250 by electroporation. HSC 72, 230 and 62 belong to FA groups A, B, and D respectively.

10^{-7}, (+/- 0.22 x 10^{-7}) a result comparable, for the SV40-driven lacZ plasmid, to the earlier experiment. Overall, electroporation did not prove to be an efficient way to transfect either the FA-B fibroblast or lymphoblast.

Stable transformation using electroporation

Based on the results of the first experiment, where the OD readings seemed high and the FA-B cells transfected at two-thirds of level of the control reading, a large scale transfection of the library was attempted using electroporation. 1.0 x 10^8 cells were electroporated in 10 different trials in 0.4 ml media. The cells were selected at 200 ug/ml hygromycin B. After three weeks it was apparent that no hygromycin B resistant cells were being selected.

Method II: Lipofection

Transient assays.

The level of lacZ expression in cells transfected with a lacZ reporter plasmids via lipofection were slightly higher than ones for electroporation. Two principle lipofection experiments were carried out looking at the effect of cell number as well as the plasmid and liposome amounts.

In the first of these experiments, HSC 72 and HSC 230 cells were lipofected (using Method A) with four different plasmids, each of which contained the hyg^r gene. HSC 72, an FA-A cell line, was used as a comparison cell line to FA-B to see if the various EBV based plasmids could be successfully maintained in these cells. The ONPG assay was performed two days post-transfection and OD readings were taken 24 hours later. The readings for HSC 230 were similar to the background signal and are not shown. The results from HSC 72 are seen in table 4 and figure 9. The expression of the pH250 plasmid was three to four times above the background signals (those of pDR2, pH200 and cells with no plasmid), a poor result given the strength of the lacZ promoter. The expression of the pH210 was indistinguishable from the background signals because of the low transfection efficiency (based on the results from pH250) and because the concentration of the pH210 used was 2.0 instead of 6.0 ug.

In the second of these experiments, two of the variables mentioned earlier, amount of plasmid and liposomes, were varied to determine the optimal lipofection conditions (using method B) on FA-B lymphoblasts.

Table 4: LacZ gene expression in FA-A lymphoblasts transfected using lipofection.

trial	Plasmid type	Plasmid amt (ug)	lipofctn amt (ug)	Measured OD	Corrected OD	AU (10^-7)	hyg. resist?
1	pH210	5	40	0.057	0.007	0.04	no
2	pH250	15	40	0.163	0.112	0.75	no
3	pH200	15	40	0.051	--	--	no
4	pDR2	15	40	0.040	--	--	yes
5	-	0	40	0.027	--	--	no

HSC 72 (FA-A) cells were transfected via lipofection with two reporter (pH210 and pH250) and two non-reporter (pH200 and pDR2) plasmids all containing the hygR gene. The ONPG assay was performed 48 hours post-transfection and the OD reading taken 24 hour after that. Subsequent quantitation of pH210 revealed the concentration to be 1/3 of original measurement. The corrected OD = OD (cell trf. with lacZ plasmid) - OD (cells trf. with pH200). Two days post-transfection the cells were also placed on hygromycin B (200 ug/ml) and selected for three weeks after which time, only cells transfected with the pDR2 plamsid yeilded resistant colonies.

LacZ GENE EXPRESSION IN FA-A LYMPHOBLASTS TRANSFECTED VIA LIPOFECTION.

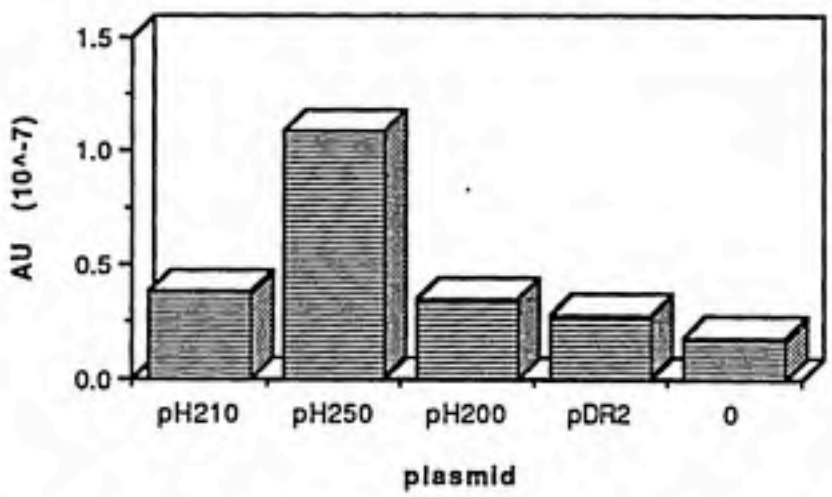


Figure 9: HSC 72 cells were lipofected with 2 ug of pH210 and 6 ug each of pH250, pH200 and pDR2 and no plasmid (O), and the lacZ expression measured 2 days later. The former two plasmids carry the lacZ gene while the latter two do not.

Table 5: LacZ gene expression in FA-B lymphoblasts transfected by lipofection.

cell number	plsmd (ug)	lipfcn (ug)	CORRECTED OD	AU*
2.50E+06	6	12	0.020	**0.10**
2.50E+06	6	24	0.048	**0.24**
2.50E+06	12	24	0.045	**0.23**
2.50E+06	12	36	0.204	**1.02**

cell number	plsmd (ug)	lipfcn (ug)	CORRECTED OD	AU*
5.00E+06	6	12	0.154	**0.77**
5.00E+06	6	24	0.083	**0.42**
5.00E+06	12	24	0.148	**0.74**
5.00E+06	12	36	0.007	**0.04**

HSC 230 (FA-B) cells were transfected with a lacZ reporter plasmid (pH210) by lipofection using two different cell concentrations. The amount of plasmid was varied as was the amount of liposome. The ONPG assay was performed 48 hours post-transfection using 2 x 10^6 cells and the OD readings taken 24 hours later. The corrected OD = OD (lacZ transfected cells) - OD (cells only).

* = absorbance units per cell x 10^-7

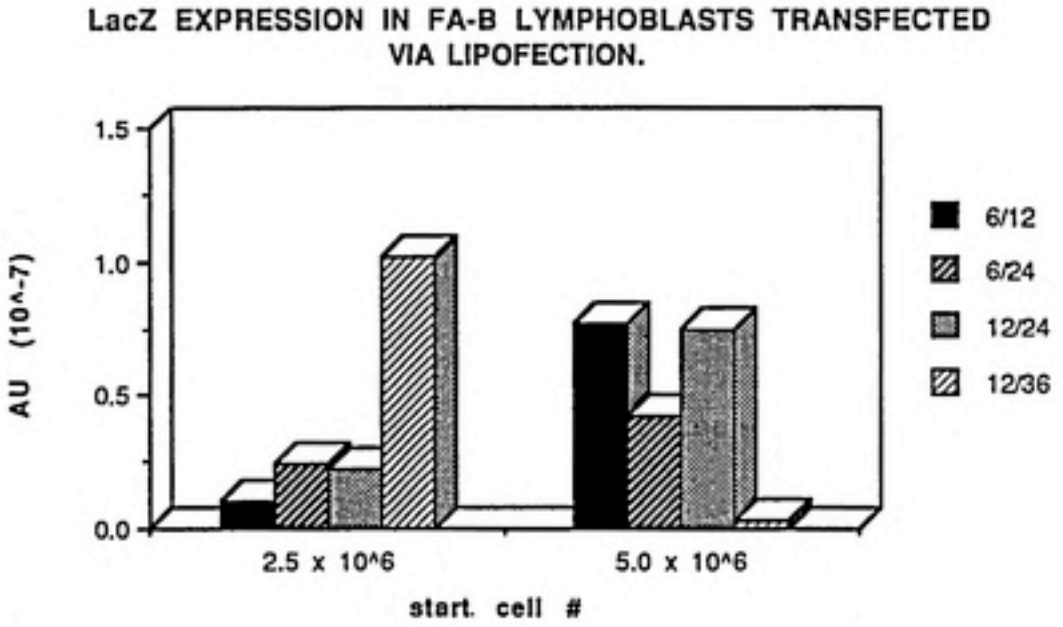


Figure 10: Two different concentrations of HSC 230 cells were lipofected with various plasmid (ug pH210) to liposome (ug lipofectin) amounts. The lacZ expression was measured 2 days post-transfection and given as AU per cell.

The results are shown in table 5 and figure 10. No clear pattern emerged as to the ideal conditions. For the four trials which started with 2.5 x 10^6 cells, the relative expression of the first three is below 0.25 x 10^{-7} AU's per cell, while the fourth condition, which used 12.0 ug of pH210 with 36.0 ug of liposome had relative expression of over 1.00 x 10^{-7}. For trials which started with 5.0 x 10^6 cells, a different pattern emerged with the first three conditions having the highest levels of expression, (AU's of over 0.70 x 10^{-7} for conditions #1 and #3 and 0.42 x 10^{-7} for condition #2) and the last one having little or no expression. (This low reading for the last condition is most likely due to an error in preparation). One reason for the disparity in the results may be that the cells in the latter trial (those starting with 5.0 x 10^6 cells) were incubated for two days post-transfection at a cell density too high to allow for the cells to double (1.0 x 10^6 cell/ml). This may have had an adverse effect on the expression of the plasmid. Though no clear pattern was seen which would indicate the best conditions, results were obtained with this method that were at least twice the order of magnitude as those obtained with transfection by electroporation

Stable transformation using lipofection.

The HSC 72 and HSC 230 cells that were lipofected in the first experiment were place on hygromycin B (200 ug/ml) 2 days post-transfection to select for hygromycin resistant colonies. After three to four weeks only HSC 72 cells transfected with the pDR2 library showed resistance to hygromycin. This is significant for two reasons. One is that the hygromycin resistance cells in the FA-A lymphoblasts were obtained despite a rather low level of transfection based on the lacZ gene expression, (AU/cell = 0.75 with CMV lacZ, comparable to 0.15 for SV40-lacZ). The same or greater results were obtained in various trials on the FA-B lymphoblast using the same plasmid yet no resistant cell could be obtained. The second is that, out of the four plasmids transfected, only the pDR2 plasmid yielded resistant cells. The reason for this may be that it is driven by a Rous-Sarcoma virus (RSV) promoter, which is known to be stronger than the Simian virus (SV40) promoter found in the other three plasmids. It also contains a promotor-less EBNA-1 gene which, if expressed, may enable it to be maintained as an episome, thus preventing its being cut (potentially at the hyg^r gene site) and integrated into the

genome, thus decreasing the number of cells expressing the hygromycin resistance gene. HSC's 72 and 230 were both transformed with EBV containing the EBNA-1 gene and thus should already express this protein. However it may be that the additional expression of EBNA-1 provided by the pDR2 plasmid may confer an advantage to these cells over cells transfected with the other plasmids. There is indeed evidence that the EBNA-1 gene of pDR2 is expressed. When plasmid DNA was extracted from an SV40-immortalized cell line carrying the pDR2 plasmid and used to transfect bacteria, plaques were formed after 24 hours in the presence of ampicillin. This would indicate that the DNA collected from these cells was pDR2, since it is the only known virus in these cells which confers resistance to ampicillin, and hence, that pDR2 was maintained as an episome which it will do only in the presence of EBNA-1 (personal observations).

Another transfection of pDR2 plasmid into HSC 230 cells by lipofection using method B was initiated and the cells selected at 100 ug/ml two days post-transfection. As with the previous experiment, no resistant cells were obtained.

Method III: Endocytosis-fection

Two experimental tests have been performed with HSC 230 cells using the endocytosis-fection method, both with markedly higher (4X) levels of transient lacZ expression than either lipofection or electroporation (figure 16). The crucial variables in endocytosis are the cell number, and the amount of adenovirus, antibody poly-lysine, transferrin poly-lysine, poly-lysine, and plasmid, as well as the order in which they are incubated (See figures 5 and 6). An additional factor which was tested in the first experiment was the induction of transferrin receptors on the cell surface by the exposure of the cells to desferrin.

Transient Results - Endocytosis-fection - Test #1:

In the initial test of the endocytotic-fection method all of the variables were kept constant with the exception that one group of cells was exposed to the drug desferrin, while the other group was not. Concurrent with the experiment on FA cells, a fellow lab partner, Dr. Suming Wang, also carried

out the same experiment on Xeroderma Pigmentosum variant (XP-V) cells *(50)*.

Effect of desferrin on cell survival.

It appears that the treatment of the cells with desferrin was extremely toxic to the cells. On the day before the transfection, both the treated and untreated plates contained 6.0 x 10^6 HSC 230 cells. The next day, 20% of the cells from each plate were used for the endocytosis-fection experiment and the remaining cells used for a electroporation experiment. Thirty hours post transfection, of the cells originally incubated with desferrin, only 16.7 x 10^5 cells remained while over 113.0 x 10^5 cells remained for the unexposed cells, over a six-fold difference in number (table 6).

Effect of transfection method on cell survival.

In addition, it appears that the endocytosis-fection treatment by itself was more deleterious to the cells than the electroporation treatment. At the time of transfection, there were four times more cells used for the latter experiment than for the former while after 30 hours the difference was 7.5 times (table 6).

Effect of Desferrin on transient transfection efficiency:

The OD reading of the HSC 230 cells treated with desferrin was similar to the background level, due in part to the low number of cells which survived to be tested. Therefor the effect of desferrin on the efficiency of transfection for HSC 230 cells could not be determined. However, the XP-V cells treated with desferrin had a lacZ level of expression 50% that of the untreated cells, (15.0 x 10^{-7} compared to 30.0 x 10^{-7} absorbance units per cell, [Wang, 1992, unpublished]).

Efficiency of transfection using endocytosis-fection.

For the cells which were not exposed to desferrin, the level of lacZ expression was higher than with any previous transfections on HSC 230 cells using either electroporation or lipofection. The level of lacZ expression in the FA-B lymphoblasts using this method was 7.6 x 10^{-7} AU's per cell, (OD reading of 0.34 for 4.5 x 10^5 cells), while for the XP-V

Table 6: Effect of desferrin on FA-B lymphoblasts.

Condition->	desf -				desf +		
	live cell numbers		TOTALS		live cell numbers		TOTALS
DAY -1			60				60
DAY 0	Endo.	Elec.			Endo.	Elec.	
	20%	80%	100%		20%	80%	100%
DAY +1	13.40	100.00	113.40		4.20	12.50	16.70

All numbers equal number of HSC 230 cells x 10^5.

On the day before the transfection experiment, approximately 60 x 10^5 cells were seeded into each of two plates, one containing 50nM desferrin (desf+). On day 0, all of the cells in each plate were split and used for either endocytosis-fection (Endo.) or electroporation (Elec.). On day 1 the cells were counted. The number of cells treated with desferrin that survived until day 1 was 15% that of the number of untreated cells.

7: LacZ gene expression in FA-B lymphoblasts tranfected via endocytosis-fection.

condition	OD at 420 nm		corrtd	Cell #	AU
	original	bkgrd level†	OD	for ONPG	(10^-7)
desf +	0.062	0.035	NAB	1.40E+05	- -
desf -	0.382	0.040	0.342	4.50E+05	7.60

HSC 230 cells, some incubated in the presense of 50 nM desferrin (desf +) 20 hours pre-transfection were transfected with the lacZ containing plamsid, pH250. An ONPG assay was initiated 30 hours post-transfection with 1.0 ml of cell suspension and maximum optical density (OD) readings were obtained 62 hours later.

† = estimated background level of lacZ expression based on previous experiments.
NAB - Not above background

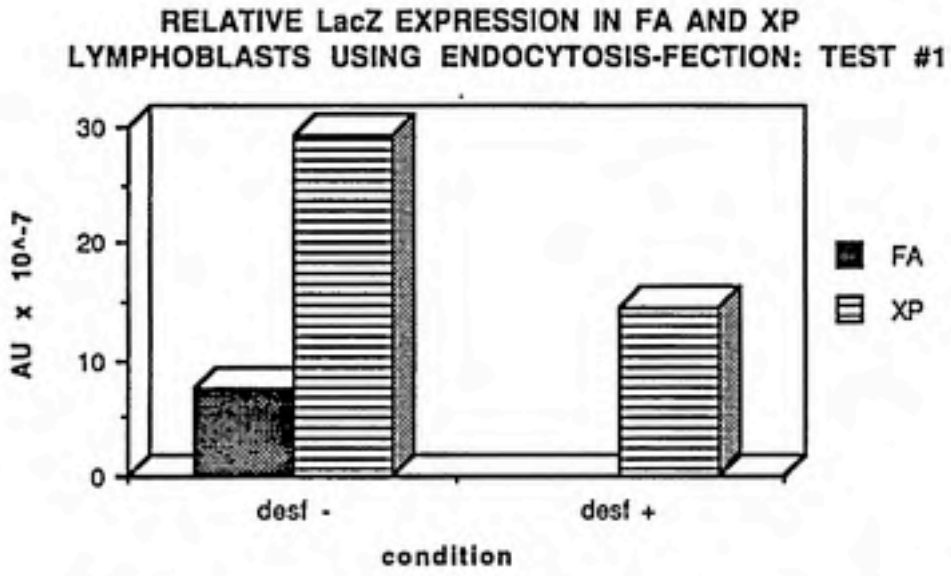


Figure 11: Relative transfection levels of lymphoblasts from Xeroderma Pigmentosum (XP) and Fanconi's Anemia (FA) using endocytosis-fection. Cells in the "desf +" condition were incubated in 50 nM desferrin 20 hours prior to the experiment.

lymphoblasts it was over 30.0 x 10^{-7}, (OD reading of 2.2 for 7.5 x 10^5 cells, [table 7, figure 11]).

Endocytosis-fection - Test #2:

A second test using endocytosis-fection was carried out on the FA-B lymphoblast cell line using the pH210 reporter plasmid. Based on findings by Dr. S. Wang that the poly-lysine 295 (pL_{295}) exerted a toxic effect on the cells, it was omitted in some of the trials of this experiment. The amount of adenovirus was also reduced in one of the trials to test whether use of a lower amount might possibly improve the chances for long-term selection. By reducing the adenovirus amount, a threshold concentration may be discovered which can be tolerated by the cells thus allowing stable maintainance of the plasmid. To this same effect, cells in trial #2 were not placed on hygromycin until four days post-transfection instead of two, thus allowing the cells time to recover from the effect of the endocytosis.

HSC 230 cells in trial #3 of this experiment were transfected under the same conditions as the cells in Test #1 with the exception of the plasmid type (pH210 instead of a CMV driven lacZ construct provided by Dr. D. Curiel), and the plasmid amount (2.0 ug instead of 6.0). The maximum reading for this condition, taken 48 hours after transfection, was 3.7 x 10^{-7} AU's percell, (OD = 0.75 for 2 x 10^6 cells). This result was over 20% the maximum level of lacZ expression given by the cells line HSC93p588Z, a normal lymphoblast which carries the pH210 plasmid (table 8, figure 12). Taking into account the differences in promoters, this result is of a greater magnitude than the results from the first test. It is also approximately four-times greater than the best results obtained by lipofection using the same plasmid.

Effect of poly-lysine 295 (pL_{295}).

The use of poly-lysine had an effect on both the cell growth and transfection efficiency. In trial #3, which used pL_{295}, there was a 56% increase in cell number after two days. This figure was under one-half as much as the over 100% increase for the other trials (table 9, figure 13). This indicates that the use of pL_{295} has a negative effect on cell growth, most likely by increasing the amount of adenovirus that entered the cells, thus increasing the toxicity to the cells. The increased toxicity to the cells corresponded to an increase in efficiency of transfection. Trial #3 was

Table 8: LacZ Gene Expression in FA-B Lymphoblasts Transfected Via Endocytosis-fection; Test 2.

A)

3 hour incubation in ONPG: Efficiency of tranfection.				
	OD at 420 nm		transf.	AU
trial	original	corrected†	effec.	(10^-7)
1	0.18	0.16	10.4%	0.79
2	0.12	0.09	5.9%	0.45
3	0.37	0.35	22.7%	1.73
4	0.07	0.05	3.2%	0.25
pc	1.55	1.53	100.0%	7.63

B)

16 hour incubation in ONPG: Max OD readings.			
	OD at 420 nm		AU
trial	original	corrected	(10^-7)
1	0.36	0.31	1.55
2	0.24	0.19	0.93
3	0.80	0.75	3.73
4	0.15	0.10	0.48
pc	2.23	2.18	10.90

HSC 230 cells were transfected via endocytosis-fection with the lacZ containing plasmid, pH210, under various conditions (See Materials and Methods) and an ONPG assay was initiated 48 hours post-transfection. The optical density (OD) readings were taken (A) after 3 hours to determine the effeciency of transfection and (B) after 16 hours which gave the maximum readings. All trials were done using standard conditions with the exceptions of Trials #1 and 2 didn't include poly-lysine 295 (pL) and Trial 4 used 1/2 standard adenovirus amount and no pL. HSC 93p588Z, a normal lymphoblast cell line containing pH210 was used in the ONPG assay as the positive control (pc), indicating 100% lacZ expression.

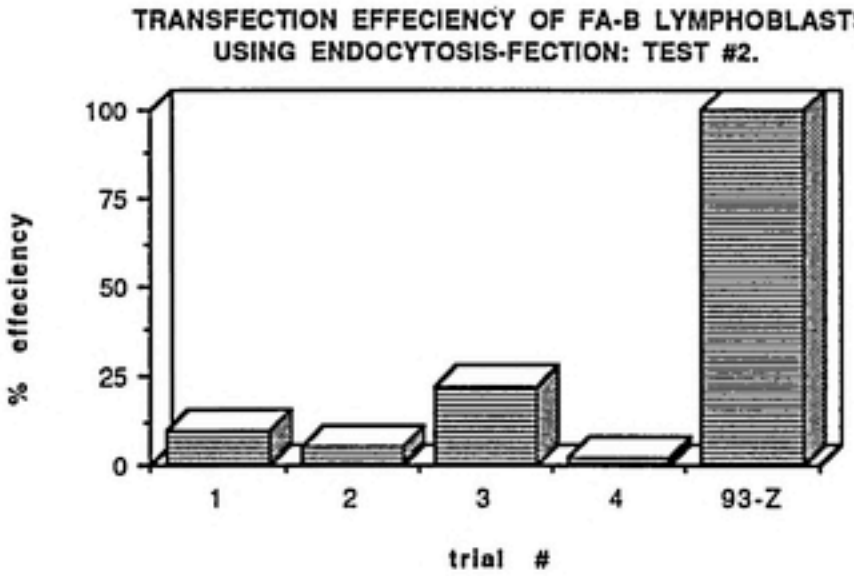


Figure 12: Effeciency of transfection of HSC 230 cells with pH210 based on the expression of HSC 93p588Z (93-Z), a normal lymphoblast cell line carrying the lacZ gene. #'s 1,2- no pL, #4, no pL and 1/2 amt. adenovirus.

Table 9. Effect of Endocytosis-fection Treatment on HSC 230 Cell Growth: Test 2.

trial #	DAY 0	DAY 2	X incrs.	% incrs.
1	40	80	2.00	100.00
2	40	85	2.13	112.50
3	40	62.5	1.56	56.25
4	40	81	2.03	102.50

40 x 10^5 HSC 230 cells were transfected on day 0 via endocytosis-fection. They were incubated in complete media for 2 days and then counted again using the trypan blue exclusion assay. Live cell numbers (given in number x 10^5) were compared with the original cell counts and the percentage growth calculated. Trials #1, 2 and 4 used no polylysine 295, while trial #3 did.

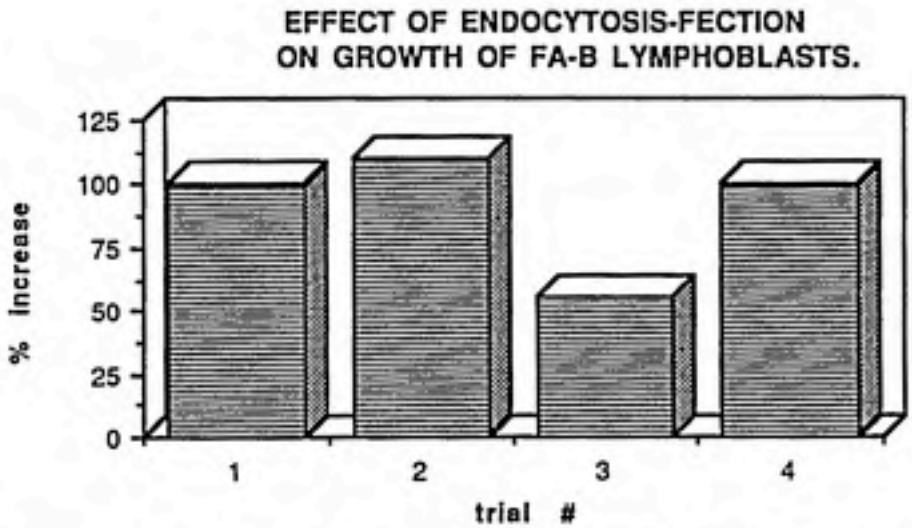


Figure 13: % increase in cell growth of HSC 230 cells 48 hours post-endocytosis-fection. Standard conditions were used except Trials #1, 2, and 4 used no polylysine while Trial #3 did. Trial #4 also used 1/2 amount of adenovirus.

transfected at 22.0% of the control compared with 10.0% for trial #1, a two-fold increase. Trial #3, as well, had a corrected AU reading of 3.78 x 10^{-7} compared with 1.6 x 10^{-7} for trial #1. This positive correlation between the level of toxicity to the cells and efficiency of transfection, in transfection procedures such as electroporation, lipofection and endocytosis-fection is well established and is confirmed here.

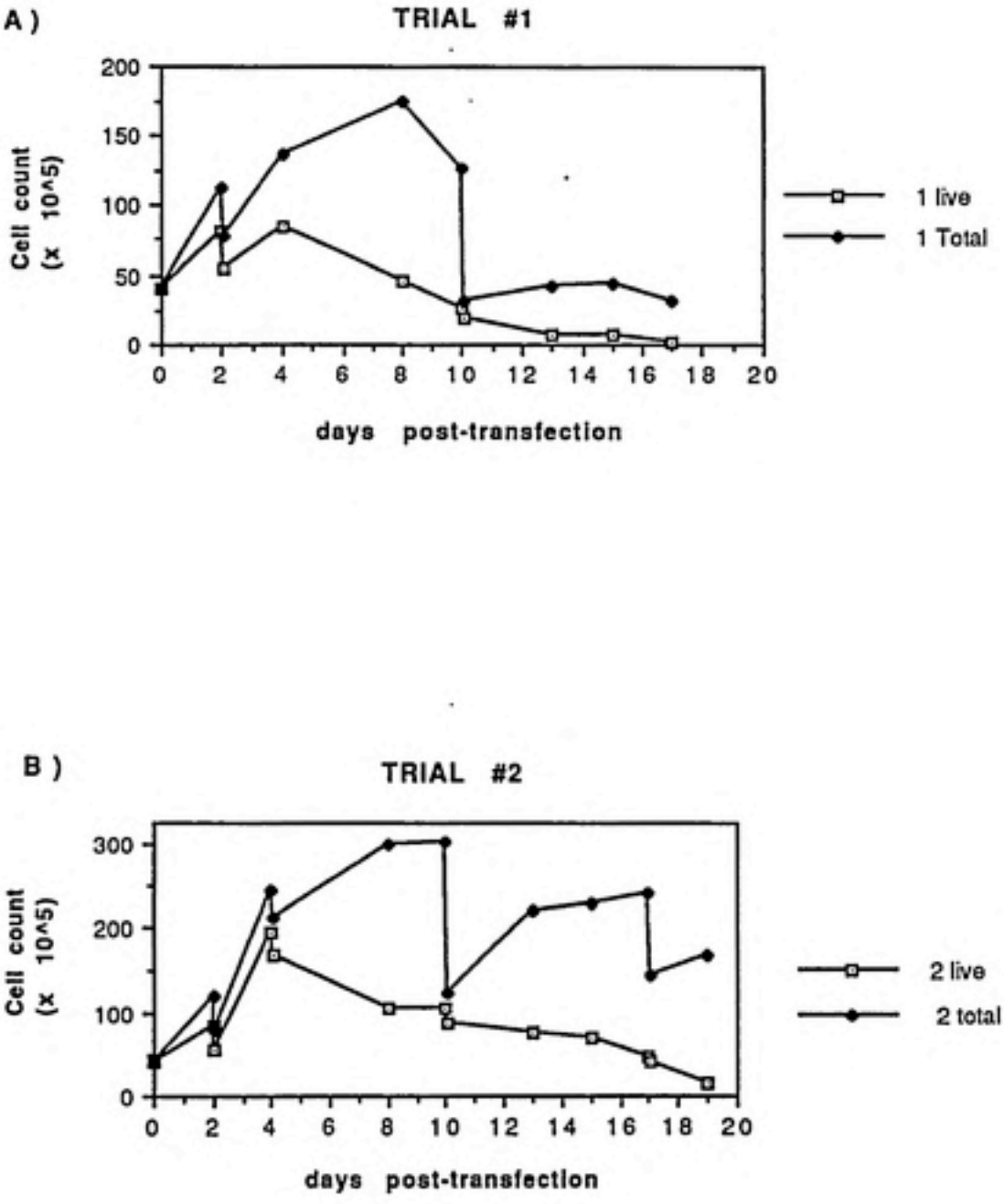
Effect of adenovirus.

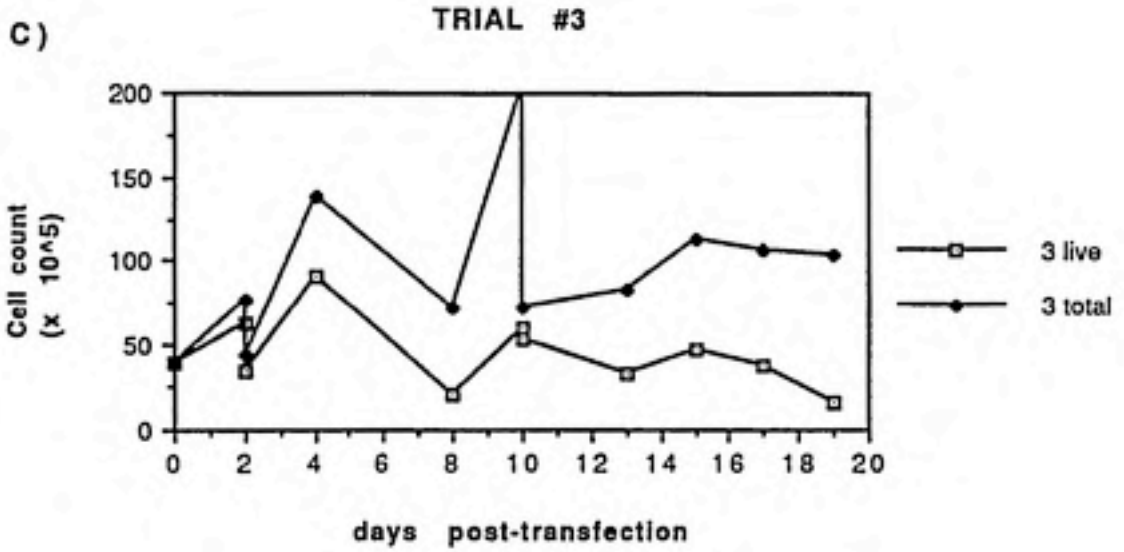
Trial #4 of the experiment used one-half of the amount of adenovirus as trial #1 as well as no pL295. There appeared to be no effect on cell growth with the decrease in adenovirus. This may be due to the lower efficiency of transfection caused by the absence of pL295. Though no effect was seen on cell growth, there was a substantial effect in transfection efficiency. The low adenovirus condition had a transfection efficiency of approximately 3.0% compared with over 10.0 % for the same conditions but with the normal adenovirus amount, a three-fold reduction.

Stable transformation using endocytosis-fection.

While results from the transient transfection using this method have yielded high levels of transfection efficiencies (Dr. Wang, unpublished results, 11, 56) few tests have been carried out on the long term maintenance of DNA transfected via endocytosis-fection. In the second of the these tests, trials #1, 3, and 4 were placed on 125 ug/ml hygromycin two days post-transfection while trial #2 was allowed two additional days to recover before it was also placed on hygromycin. The results of the selection are found in figure 14. The cells were counted every two days and the media was changed every 4-5 days. After day 4 there appears to be a slow but steady decrease in the number of live cells. After ten days the dead cells were removed over a ficoll cushion. After 22 days very few live cells remained and the experiment was aborted. The failure to achieve resistant colonies may be the result of the toxicity of the adenovirus to the cells, or it could be that the level of EBNA-1 expression in these EBV transformed cells is too low to maintain the pH210 plasmid as episome thus leading the loss or integration of the plasmid. In an alternate experiment, the pH250 plasmid was transfected via endocytosis-fection into both an XP-V fibroblast and lymphoblast cell line and high

FIGURE 14: SELECTION OF HYGROMYCIN RESISTANT FA-B LYMPHOBLASTS TRANSFECTED VIA ENDOCYTOSIS-FECTION.





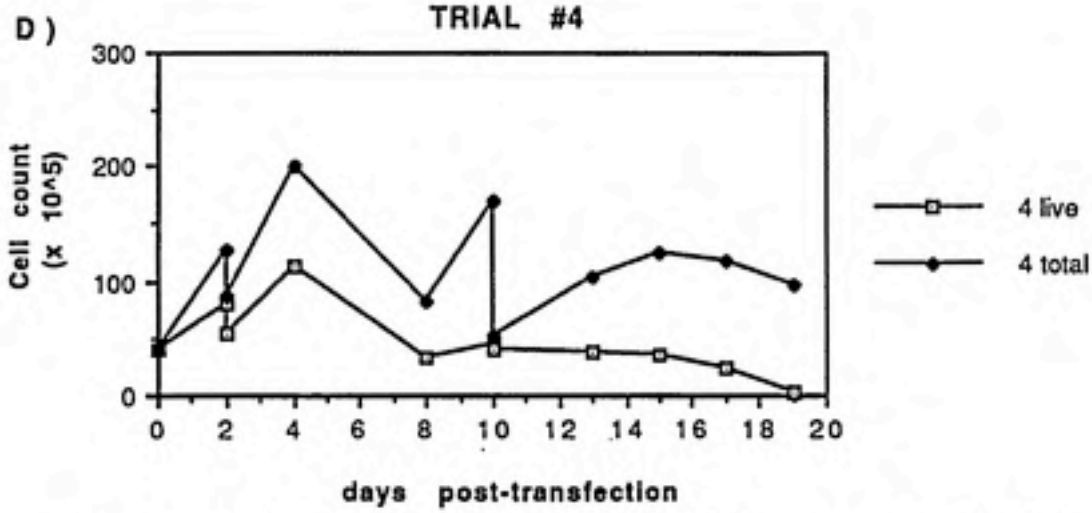


Figure 14: Selection for hygromycin resistance in HSC 230 cells transfected via endocytosis-fection with pH210. Hygromycin B (100 ug/ml) was added 2 days post-transfection except trial 2 (B) which was placed on hygromycin after 4 days. Trials 1 and 2, (A and B), were transfected under the same conditions, (with no pL), trial 3 (C) used standard conditions and trial 4 (D) used no pL and 1/2 amount of adenovirus.

transientlevels of expression were seen. However when placed on selection, no resistant colonies were obtained (Dr. S. Wang unpublished data).

SENSITIVITY ANALYSIS OF SELECTING AGENTS

One of the most important factors in finding the FA gene is the selection, first of cells that are resistant to hygromycin B, and then to cells which are resistant to the effect of DNA cross-linking agents. In order to do this, the proper concentrations of the selecting agents must be found, concentrations that are strong enough to kill the majority of the cells but not strong enough to kill even those cells which take-up the resistant-bearing plasmid. The concentration that was chosen for most of the selection procedures is that which inhibits cell growth to 1-10% of normal after 6 days.

Hygromycin B.

In the two major tests for hygromycin sensitivity in HSC 230 cells the effective concentration which inhibited growth to "x" percent of normal (ECx) were; EC 50 = 30 ug/ml, EC 10 = 100 ug/ml and the EC 1 = 120 ug/ml. 100-125 ug /ml were used to select for plasmids transfected into HSC 230 cells (figure 15).

MMC sensitivity.

Figure 16A shows the sensitivity to MMC of four FA lymphoblasts cell lines as well as two normal lymphoblasts cell lines. These confirm what has been shown before, namely that, of the FA cell lines, HSC 62 (FA-D) is the least sensitive among the four FA cell lines, having a sensitivity close to that of the normal cell line HSC 93 (the former has an EC10 of 55-65 ng/ml while the latter has one of over 100 ng/ml, a 2-4 times difference), that HSC's 72 and 99 (FA-A) cell lines have an intermediate sensitivity which are similar to each other, and finally that HSC 230 (FA-B) is the most sensitive (27, 51). The EC 10 for HSC 230 is around 10 nM. This is the concentration that will be used when the library is established (table 10).

PUVA sensitivity:

Figure 16B shows the sensitivity to HMT and UVA light (PUVA) of the six aforementioned cell lines. The results are in line with other sensitivity studies done using PUVA on FA cells *(39)*. The FA cell lines show a similar pattern of sensitivity relative to each other as was the case with MMC,

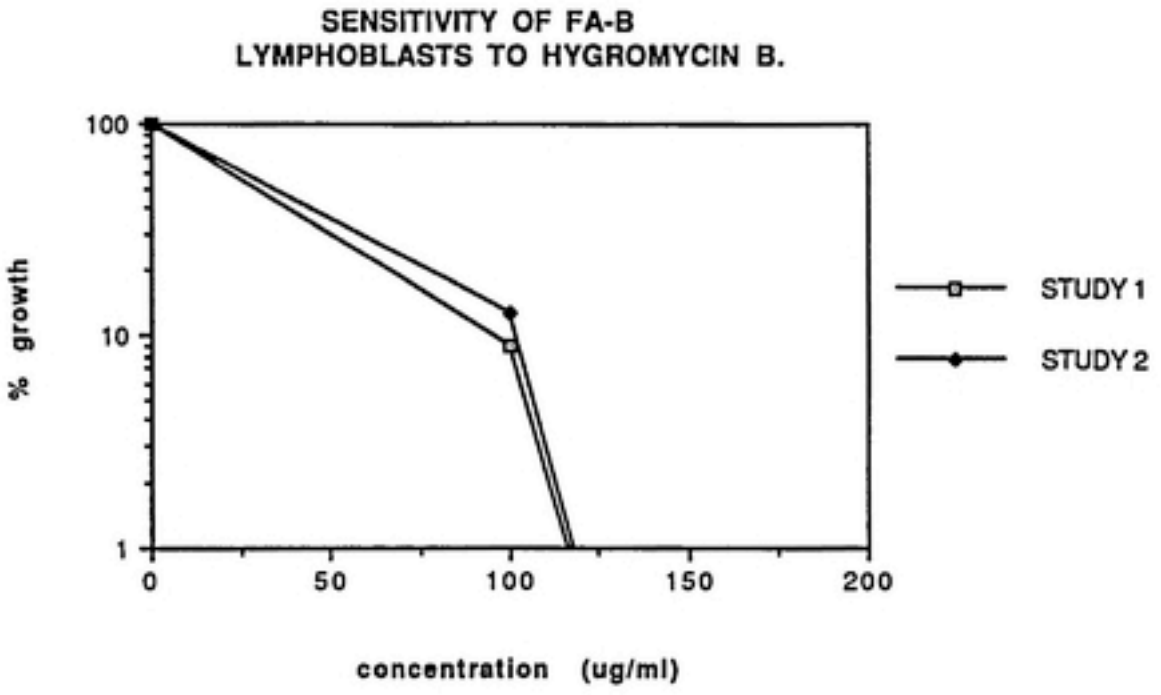


Figure 15: Effect of hygromycin B on growth of HSC 230 cells in two independent studies. Cells were initially exposed to hygromycin B on day 0 and then counted on day 6.

Table 10: Effect on Growth of FA Lymphoblasts Treated with DNA Cross-linking Agents.

(A) MMC Sensitivity

CELL LINE	GROUP	EC 50	EC10
UC	Normal	CBD†	CBD
HSC 93	Normal	26	CBD
HSC 72	FA-A	< 4.00	45.00
HSC 99	FA-A	< 4.00	55-65
HSC 230	FA-B	< 4.00	10.00
HSC 62	FA-D	12.00	55-90

(B) PUVA Sensitivity

CELL LINE	GROUP	EC 50	EC10	EC1
UC	Normal	CBD†	CBD	CBD
HSC 93	Normal	10	55	75
HSC 72	FA-A	1.00	2.00	3.50
HSC 99	FA-A	1.00	2.00	3.50
HSC 230	FA-B	< 0.25	< 0.50	< 0.50
HSC 62	FA-D	1.50	3.50	7.00

Table 10: Sensitivity of four FA cell lines to the cross-linking agents; (A) mitomycin C (MMC) in nM and (B) psoralen and UVA light (PUVA) in ng/ml. 1.0 and 2.0 x 10^5 cells respectively for A and B were seeded into each well of a 24 well plate. Each column was then treated with progressively higher doses of the cross-linking agent. ECx = effective concentration needed to reduce cell growth to "x" % of normal. The UC cell line is not sensitive to the cross-linking agents at the concentrations administered, therefore the EC50 and EC10 could not be determined, (CBD).

SENSITIVITY OF FA LYMPHOBLASTS TO DNA CROSSLINKING AGENTS.

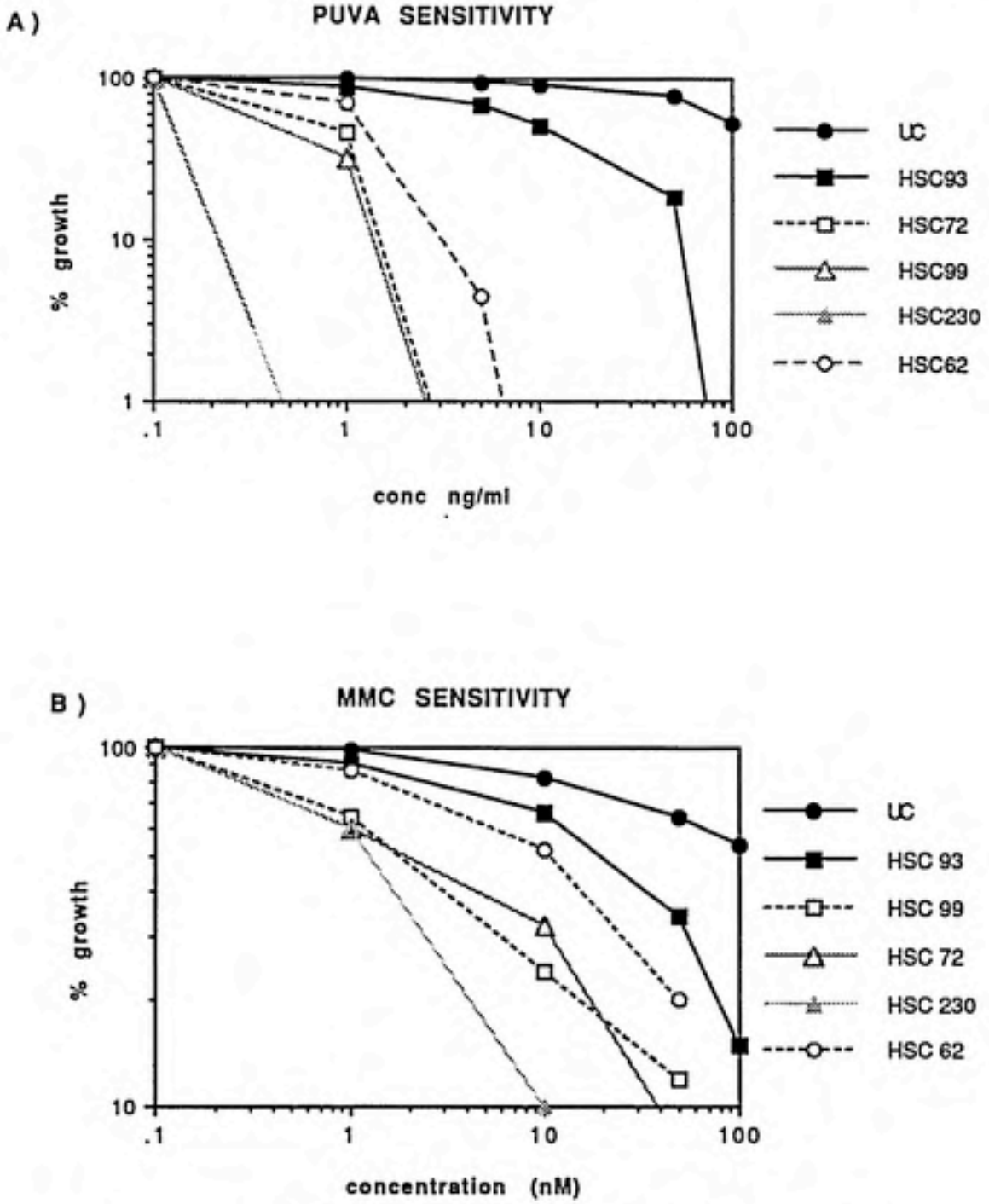


Figure 16: Sensitivity of four FA lymphoblasts to; (A), HMT (psoralen) and UVA light (PUVA), and (B) to mitomycin C (MMC). UC and HSC 93 are normal lymphoblasts while HSC's 72, 99, 230, and 62 belong to FA groups A, A, B, and D respectively.

though they, as a whole, exhibit a much greater sensitivity than with MMC relative to the normal cell lines (table 10). The EC10 of HSC 62, the least sensitive of the FA cell lines, is approximately 4.5 ng/ml while the EC10 of the most sensitive normal cell line was over 65 ng/ml, almost 15 times greater. There was only, at most, only a four times difference between the sensitivities of HSC 62 and HSC 93 to MMC. The FA-B cell line showed extreme sensitivity to PUVA treatment having an EC 10 of under 0.5 ng/ml.

DISCUSSION

In the quest to discover the most efficient method to transfect an entire cDNA library into FA-B cells, three different transfection methods were explored including electroporation, lipofection and endocytosis-fection (figure 17). While transient results were obtained which would indicate that stable transfection could take place, this result failed to occur.

Electroporation gave consistently poor results and was dropped from consideration. Using lipofection, lacZ expression readings of over 1.0×10^{-7} AU's per cell were obtained using the SV40-driven lacZ plasmid (pH210) on the FA-B lymphoblast cell line, HSC 230. This number is 2-3 times higher than the best result obtained via electroporation and four times less than lacZ expression levels obtained via endocytosis-fection. With a lower lacZ expression, (0.15×10^{-7} AU's per cell), the pDR2 plasmid was successfully transfected, using lipofection, into an FA-A lymphoblast cell line, HSC 72. However, as mentioned, this result has not been duplicated in the FA-B cell line. This result would indicate cell line differences in stable transfection efficiencies, with the FA-B lymphoblast cell line apparently needing a higher level of DNA-transfer in order to stably maintain the plasmid. The reason for this may involve the inherently greater genetic instability of FA lymphoblasts from group B as compared to group A as seen in the cross-link sensitivity analysis and chromosome aberration studies. It may also concern the fact that the FA group B lymphoblast (HSC 230) grows at a slower rate than does those from group A (HSC's 72, and 99). It should be noted that the group C lymphoblast cell line, HSC 536, which has been cloned, though genetically the most unstable of the established and characterized FA lymphoblast cell lines, has the highest rate of growth among each of these lines, [*52, personal observation*]) .

The highest efficiency of transfection of the FA-B lymphoblast cells, based on expression of the lacZ gene, was found using endocytosis-fection. Using the pH210 plasmid, an expression level of about 4.0 x 10^{-7} AU's per cell (approximately a 20% transfection efficiency) was obtained. This number is almost four times higher than the best result obtained using lipofection. However, no resistant colonies were obtained when these cells were placed on hygromycin. The failure to achieve a stable transformation of the FA-B cells as well as the XP cells transfected with either pH210 or pH250 could be due to either the nature of the plasmids and/or of some component of the endocytosis-fection method.

The former hypothesis is supported by the fact that when two different cell lines, one fibroblast and one lymphoblast, were transfected with the cDNA library pDR2, (containing the EBNA-1 gene and RSV promotor), along with the mini-EBV lacZ plasmids pH210 and pH250, only lymphoblast cells receiving the cDNA plasmid became hygromycin resistant, while in the fibroblast, resistant colonies were obtained with the lacZ constructs, though at a much lower number than with the pDR2 (*personal observation*).

There are both experimental and theoretical support for the latter hypothesis. Experimentally, the lacZ construct pH250 was transfected at a high efficiency (as determined by the ONPG assay) in human XP cells via endocytosis-fection and then placed on selection in hygromycin, yet no resistant colonies were obtained. This failure, as well as the one with the FA-B lymphoblasts, may be due to the toxicity of the adenovirus to the cells. When the adenovirus is rendered replication defective by psoralen and UVA light, its titer is not totally abolished*(11, 12)* and replication of the adenovirus at a high enough level would be lethal to the cells. Adenovirus's are also known to recombine with endogenous cell virus's to once again become replication-competent. Additionally, it has been reported that the penton protein found in the capsid of the adenovirus is also toxic to mammalian cells *(9)*.

Alternately, the great amount of endocytosis that invariably occurs following the binding of the adenovirus-DNA complex to the numerous adenovirus receptors on the surface of many cells, may be a shock to the cell system too great for the cells to handle and thus may lead to cell death.

In the effort to achieve the most efficient transfection protocol using endocytosis-fection a few experiments were attempted. In the first experiment, the lymphoblast cells were exposed to the compound desferrin, which induces the production of transferrin receptors on the surface of many cells *(11, 12, 58)*. In many monolayer cell lines this action resulted in an increase in transfection efficiency. However, the desferrin, at the dose given, proved toxic to the FA and XP lymphoblast cells and so whose result could not accurately be determined.

In another of the experiments, the poly-lysine 295, which is added to the DNA-Adenovirus conjugate after the DNA and which, along with the transferrin poly-lysine causes the folding of the DNA into a "doughnut shape", was omitted three of the trials (figure 6). This resulted in two-fold decrease in the lacZ expression underlying the importance of this component to the process.

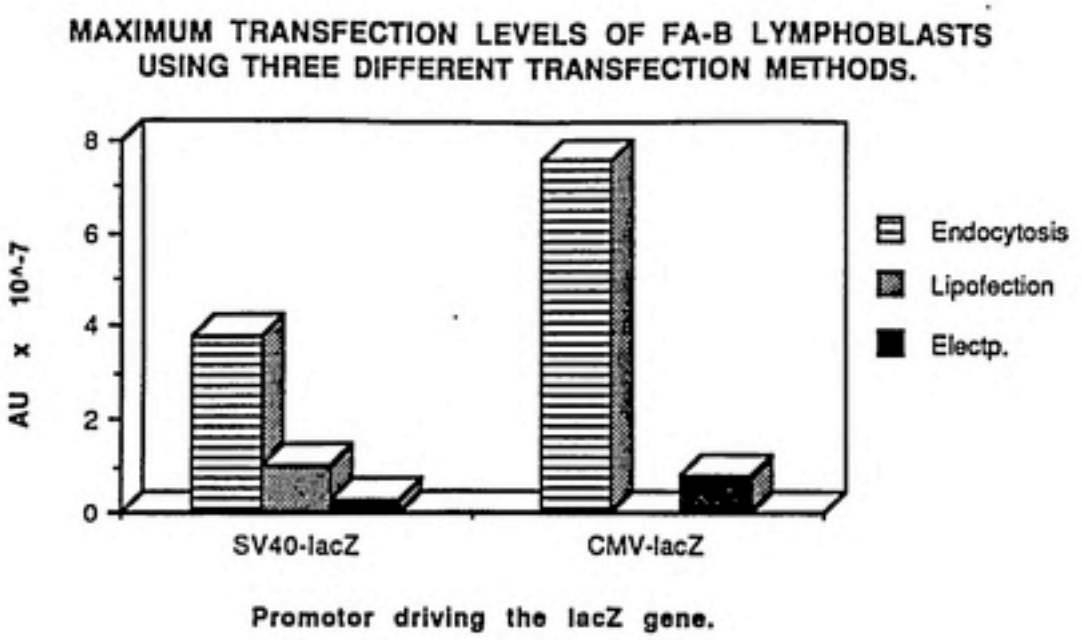


Figure 17: HSC 230 (FA-B) cells were transfected via electroporation, lipofection and endocytosis-fection, with reporter plasmids driven by two different promotors.

CHAPTER VII: CONCLUSIONS AND RECOMMENDATIONS-PROJECT 2

The objectives for the first part of this study, to determine the most efficient method of transfection for cells belonging to Fanconi's anemia group B cells, was partially achieved, though more work remains to be done.

The FA-B lymphoblastoid cells have proved resistant to the uptake and stable maintenance of certain plasmids transfected through electroporation, lipofection, and endocytosis-fection. Transiently, the efficiencies of transfection by electroporation have proved too low to be feasible as a choice to insert the cDNA library, though selection for long term maintenance of transfected plasmids was tried and failed in these cells using this method. Transfection by lipofection has yielded better transient results than with electroporation though stable transformation of FA-B lymphoblast cells has also proved elusive. The FA-A lymphoblastoid cell line, HSC 72, however, was able to be stably transformed with the pDR2 cDNA library using lipofection.

A novel transfection system, endocytosis-fection, was found to yield a four times greater signal in HSC 230 cells than with the other methods. Though transient transfection efficiencies are high, questions remain as to whether a plasmid transfected by this method can be stably maintained as well as can any plasmid be maintained in this specific cell line. In at least two transfection experiments involving FA and XP cells, selection of the transfected plasmid could not be achieved despite high transient level of expression of the plasmid. Alternatively, while resistant colonies were obtained in an FA group A lymphoblast cell line at a transient lacZ expression level of approximately 0.15 x 10-7 AU's/cell, using lipofection, no such resistance could be obtained in the group B lymphoblasts despite levels of expression almost 4-5 times higher than this.

The insertion of a cDNA library into FA cells stands as the major obstacle in the cloning of the gene. Besides a complete cDNA library, a cell line which can take up plasmid DNA and an efficient method of transfection are also needed. The only fully transformed FA-B

lymphoblast cell line that has been characterized (HSC 230) has proved resistant to the uptake of foreign DNA using three different methods of transfection. Using the novel adenovirus-assisted, endocytosis-mediated system of transfection, plasmid DNA can be transferred into these cells at a relatively high rate though once in the cells, the DNA can not be maintained. This may indicate a deficiency with this transfection system as it presently stands though modifications have been proposed which may overcome this deficit. Once a library has been successfully been established in a FA cell line, selecting for the gene is a relatively straightforward procedure, though success in this venture also depends on the quality of the library and the presence of the intact FA gene among the cDNA. If efficient ways can be found to insert the library into the FA-B cells, then it is a matter of time before the gene will be cloned.

Recommendations for Future Research.

The endocytosis-mediated method of transfection may be the most promising system to achieve the goal of the insertion of a cDNA library into FA-B lymphoblast cells, and hence to progress further in the cloning of the FA-B gene if modifications can be found which render the method less toxic to the cells. It remains to be seen if a plasmid transfected in this manner can be stably maintained in the FA-B cell line. In order to test this out, the following experiments should be performed.

First, HSC 230 cells should be transfected with the pDR2 cDNA library and pH210 using endocytosis-fection under the same conditions that were used in trial #3 in test #2 of the endocytosis-fection experiments, and placed under selection with hygromycin B, (if the transient signal was comparable to the earlier levels). If resistant colonies can be selected, then this would demonstrate that plasmid DNA can be transfected and stably maintained in a cell line using the endocytosis-fection method. It would also show that the previous failure of selection using pH210 was at least partially due to the nature of the plasmid and not solely on the method of transfection. If this were the case then steps would be taken to establish the whole cDNA library into these cells.

If resistant colonies could not be selected, even after high transient lacZ expression and use of the pDR2 plasmid, this would indicate that some portion of the experiment was toxic to the cells, either the large amount of

endocytosis that occurs from the massive binding of the adenovirus or of some component of the adenovirus itself. One way to test the former hypothesis would be to repeat the experiment with both a lymphoblast and a fibroblast cell line known to transfect easily (such as the XP-V cell lines used by Dr. S. Wang), using the pH210 and pDR2 plasmids decreasing the amount of conjugate used, in the hopes of finding an optimal concentration that yielded a relatively strong lacZ signal and hygromycin resistant cells. If decreasing the amount of conjugate (and hence amount of adenovirus) resulted in a increased number of hygromycin resistant cells, this would support the former hypothesis. If no resistant colonies could be obtained, even using low amount of conjugate, this would support the latter hypothesis.

Recent endocytosis-mediated transfection experiments have been performed using a chicken-derived adenovirus (CELO) as a substitute to the human adenovirus. Positive results have been found both in the short term expression of reporter plasmids but also in long-term selection of plasmid DNA (personal communication, Dr. Cotten). Use of this new adenovirus should be pursued to see if the obstacle encountered to date can be overcome.

With the knowledge that fibroblasts are more efficiently transfected using lipofection or endocytosis-fection than are lymphoblastoid cells, transfection work should also be continued on the FA-B partially transformed fibroblast, HSC1199-11-2 to see if hygromycin resistant colonies can be established using the pDR2 library transfected via either lipofection or endocytosis-fection (with either human or chicken adenovirus). Also, if the cloning of the FA group B gene is to be cloned then steps should be taken as well to locate alternate cell lines which are more transfectable, preferable fully transformed fibroblasts, which are, in general easier to transfect (with such methods as lipofection) than are lymphoblasts.

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